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EDITED BY

W. H. PEARSALL

D.Sc., F.R.S.

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University College, London

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Nutritional and Other Aspects of Fruit-body Production in Pure Cultures of *Collybia velutipes* (Curt.) Fr.

BY

B. E. PLUNKETT

(Birkbeck College, London)

With Plates V and VI and five Figures in the Text

ABSTRACT

Fruit-body production of *Collybia velutipes* on a synthetic medium consisting of minerals, asparagine, sucrose, and vitamin B₁ has been studied. Compared with others tested this medium favoured fructification rather than mycelium growth. Primordia arose on cultures between pH 5.2 and 7.2 approximately. Relatively low initial concentration of asparagine, high initial concentration of sucrose, and increases in volume (without essential increase of the air interface) of media resulted in enhanced dry weights of sporophore crops. These factors variously affected mycelium yields and the times of primordium production. Details of growth, sporophore production, maximum yield, and changes in the medium are given.

MANY investigators have observed fruiting in pure cultures of hymenomyces, but experimental studies of fructification have been few compared with those in other major groups of fungi.

There appear to be no previous unequivocal records of sporophore formation on chemically defined media.

In nature *Collybia velutipes* causes a soft white rot of the sapwood of elm, willow, and perhaps other trees. Fruit-bodies have appeared in pure cultures upon blocks of various timbers (Costantin and Matruchot, 1894; Biffen, 1898; Falck, 1902), upon enriched sawdust (Badcock, 1943), and upon certain agars (Lutz, 1925; Hawker, 1942; Cartwright and Findlay, 1946, p. 183).

A striking feature of the sporophores is their capacity to continue spore liberation (Buller, 1924) and even to grow at temperatures in the region of 0° C. (Biffen, 1898; Stewart, 1918).

A classical study of the biology of this fungus (Biffen, 1898) was concerned principally with features of the rot caused in *Aesculus* blocks and with the developmental anatomy of the fruit-body. Biffen noted that light-starved fructifications were slender and lacked normal pilei. The passage of a stream of moist, filtered air over wood-block cultures had only the effect of delaying sporophore formation.

Thiamin (or its thiazole moiety) has been found essential for vegetative growth in *Collybia velutipes*, while pyridoxine, inositol, biotin (Barnett and

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Lilly, 1947a; Marczynski, 1943), and riboflavin (Marczynski, 1943) were inactive. Marczynski (1943) suggested the existence of other, undetermined, growth-promoting substances active in low concentration. It has been claimed that the concentration of glucose optimal for fruit-body production in *Collybia velutipes* (and other hymenomycetes) is a function of the amount of thiamin supplied to the culture (Hawker, 1942). Carbon balance-sheets for the mycelium growing on glucose-urea media have recently been prepared (Whitaker, 1951). Aeration affected the final carbon distribution.

In the present communication the effect of the concentration of certain components of a defined medium upon the growth and fruiting processes is described.

MATERIAL AND METHODS

The isolation of *Collybia velutipes* used had been kept for several years in laboratory culture upon 2 per cent. malt agar.

By comparison with wild sporophores those produced in pure culture are smaller and have rather longer stipes in relation to the cap diameter. The dark-brown coloration of the stipe, so conspicuous in nature, is less marked. The fungus was stable in appearance and physiological characteristics. A few comparisons with other isolates showed no differences.

Standard inocula 2.5 mm. square were taken at approximately the same distance behind the growing-margin of vegetative, Petri-dish cultures on thin 2 per cent. malt agar.

The medium used in most experiments was wholly 'synthetic' and, so far as macronutrients are concerned, chemically defined. It had the following composition:

Sucrose	.	.	.	.	.	.	.	.	various amounts
Asparagine	.	.	.	.	.	.	.	.	"
Magnesium sulphate ($\text{MgSO}_4, 7\text{H}_2\text{O}$)	.	.	.	.	.	.	.	.	2.5 g.
Neutral potassium phosphate (K_2HPO_4)	.	.	.	.	.	.	.	.	5.0 g.
Ferrous sulphate ($\text{FeSO}_4, 7\text{H}_2\text{O}$)	.	.	.	.	.	.	.	.	trace
Copper-distilled water	.	.	.	.	.	.	.	.	up to 1,000 ml.

The chemicals were standard laboratory reagents, when possible of the 'Analytical' standard of purity. The hydrogen-ion concentration of the medium was adjusted to pH 5.8 using N_1 hydrochloric acid. Twenty-millilitre portions of the medium were dispensed into 150-ml. conical flasks plugged with cotton wool. One millilitre of a solution of thiamin hydrochloride (1 mg./100 ml.) was then added to each flask. Sterilization, which had a negligible effect upon hydrogen-ion concentration, was by autoclaving at 15 lb. per sq. in. for 15 minutes. The 1 ml. of water introduced with the vitamin is disregarded in stating concentrations.

Following inoculation cultures were placed in a specially constructed incubator in which illumination was provided by fluorescent-tube lamps suspended above the glass top. The temperature of incubation was $20 \pm 1.5^\circ \text{C}$. with an illumination of about 8 metre candles at the culture surface.

Evaluation of sporophore production in hymenomycetes presents special

difficulties. Size variation makes the fruit-body count, used in other groups of fungi, unsatisfactory. In all the present experiments sporophore numbers were determined, but proved valueless as a fruiting-index due to the great variability of samples. Some workers have also taken into account linear dimensions or surface area (Hawker, 1942; Voderberg, 1948 et seq.), but, besides being laborious for many replicates, it is possibly inaccurate when numerous small fruit-bodies are involved. The fresh weight of sporophores has been used in researches on the cultivated mushroom (Treschow, 1944), but as some hymenomycete sporophores may retain variable amounts of water in the intercellular spaces (Stewart, 1918; Buller, unpublished researches), this also may have disadvantages. In the present work yield was assessed as dry weight of sporophores.

Harvesting was usually done when the last fruit-bodies had completed their expansion (at approximately 40 days). The cultures were tipped into an open dish and the sporophores dissected away from the mycelium with fine forceps and scissors. Any experimental error in this separation may be regarded as negligible in terms of dry weight. Mycelium and sporophores were drawn into separate tared scintered-glass crucibles, washed by a standard procedure, dried to constant weight at 80° C., cooled in a desiccator over calcium chloride, and weighed to the nearest milligram on a precision balance.

As a measure of dispersion the standard error of the mean (ϵ) of samples is given, having been calculated from the formula:

$$\epsilon = \sqrt{\left(\frac{\Sigma(m - M^2)}{N(N - 1)} \right)}.$$

When it was desired to compare the means of two samples the method recommended by Fisher (1934, p. 120) for small samples was used. The probability of two samples belonging to the same population is given in the value P . Significance in differences was assumed at the 5.0 per cent. level. The size of samples is indicated elsewhere.

During the developmental period daily observations on the course of growth of fruit-bodies were made. In the interests of easy comparison three arbitrarily selected stages were recognized. These were: stage 1, the first appearance of a tiny brown primordium on the surface of the mycelium (Pl. V, Fig. 1); stage 2, the first visible differentiation of a cap on the tiny stipe-shaft (Pl. V, Fig. 2); and stage 3, the first appearance of a flattened pileus (corresponding to the last stages of spore liberation) (Pl. V, Fig. 3, represents a sporophore a few hours before stage 3 was reached). The cap subsequently becomes reflexed and the fruit-body collapses after about 2 days. The stages apply to the first fruit-body maturing in a culture. The remaining developing sporophores ripen a few hours, or at most days, afterwards. Usually a few fruit-bodies abort, but very rarely the one first formed. After a little experience no difficulty was encountered in defining the stage of development and the large differences encountered could not be due to the personal factor.

THE CONTROL OF ILLUMINATION

In order to avoid the limiting action of the light factor, preliminary tests of its influence were made. Light-tight cardboard boxes were placed in the light incubator. Bromide papers in these boxes, at the external light intensity indicated, gave negative tests after 24 hours. By manipulation of the box-lids different daily periods of illumination at 8.0 m.c. were arranged for several batches of cultures on the synthetic medium (sucrose 7.5, asparagine 0.1 g./100 ml.). The construction of the boxes makes it improbable that conditions of aeration account for the results obtained.

Under these conditions dry weights of sporophore crops were significantly higher ($P = < 0.01$) with 0.5 or 6.0 hrs. illumination *per diem* than with 0.0. Eighteen hours caused a further increment as compared with a 6-hour light period ($P = < 0.01$). Continuous illumination was without further effect (Table I). Cultures in continuous darkness produced highly clustered and delicate fructifications with minute pilei (Pl. V, Fig. 4), suggesting that primordium and stipe production are independent of light. Transfer of such cultures to light during the developmental period promoted the further growth of the pileus. With 0.5 hr. illumination *per diem* sporophores were noticeably less robust than with longer periods and flattening of the pileus was not attained.

TABLE I

Effect on Dry Weight of Sporophores produced (in mg.) of Various Daily Periods of Illumination

Incubation time 39 days				
Daily period of illumination (in hrs.)				
0	0.5	6.0	18.0	24.0
8 ± 4.0* (6)†	43 ± 6.4	53 ± 8.0	93 ± 12.6	93 ± 18.2

* Mean ± standard error of mean.

† In this and subsequent tables the number of replicates is indicated in parentheses.

Continuous illumination was adopted as a convenient means of ensuring that the light factor should not be limiting in experiments concerned with other variables.

NUTRITION

1. *The choice of medium*

Certain preliminary trials, most of them under uncontrolled-light conditions, preceded the adoption of the synthetic medium. Fruiting occurred on 2 per cent. malt extract agar after about 5 weeks, but neither synchronously nor in all replicates.

Experiments with media which were essentially variants of the synthetic medium showed that mycelium growth (estimated visually) was very slight indeed in the absence of thiamin. This observation of partial or complete

thiamin heterotrophy represents a confirmation of the results of other workers (Marczynski, 1943; Barnett and Lilly, 1947a). Potassium nitrate was apparently not utilized as a nitrogen source even in the presence of thiamin, although an equivalent amount of nitrogen as asparagine (0.5 g./100 ml.) supported a fair growth. Sucrose and glucose (5.0 g./100 ml.) were, from colony appearance, indistinguishable as carbon sources. Being less subject to caramelization during sterilization at the temperature and hydrogen-ion concentration employed, sucrose was used in subsequent experiments.

The effect of adding certain natural extracts to the synthetic medium. Upon the synthetic medium mycelium growth appeared less abundant than upon malt extract agar (2 g./100 ml.). If a rich yeast extract was added to the medium enhanced mycelium yields resulted, but sporophores appeared later and not in all cultures. Likewise the addition of 1.5 or 2.0 g./100 ml. of certain samples of agar caused the inhibition of fruiting, although growth was abundant.

More critical results of a similar kind apply to the addition of malt extract. Here the incubation conditions were those described under 'Methods', light conditions not limiting. Groups of cultures on the standard medium (sucrose 5.0, asparagine 0.5 g./100 ml.) with the addition of 0.0, 0.0001, 0.001, 0.01, 0.1, and 1.0 g. of malt extract per 100 ml. were prepared. Cultures were harvested when 36 days old. The yields are shown in Table II.

TABLE II

The Effect upon Mycelium, Sporophore, and Total Yields (in mg.) and upon Final pH of adding Various Amounts of Malt Extract to the Synthetic Medium

	Incubation time 36 days. Amount of malt extract added (g./100 ml.).						2% malt ext. only
	0.0	0.0001	0.001	0.01	0.1	1.0	
Sporophores	94.8 ± 16.4 (5)	124.4 ± 12.8	93.6 ± 16.1	97.2 ± 12.2	31.6 ± 8.7	0	4.4
Mycelia	78.0 ± 6.2	60.8 ± 7.6	88.8 ± 16.6	87.0 ± 17.3	150.0 ± 18.0	269.8 ± 8.8	74
Total	172.8	185.2	182.4	184.2	181.6	269.8	78.4
Initial pH	5.8	5.7	5.8	5.8	5.7	5.6	4.6
Final pH	6.5 (3-4)	6.0	6.1	6.6	6.5	8.0	4.0

The addition of amounts of malt up to 0.01 g./100 ml. was without significant effect upon mycelium or sporophore yields. A sudden highly significant drop in fruit-body yield ($P = < 0.01$) and rise in mycelium dry weight ($P = 0.05-0.02$) became apparent with 0.1 g./100 ml. of added extract. Ten times as much malt extract further enhanced mycelium production ($P = < 0.01$), and sporophore yield fell to zero in all replicates. It is of interest to note that the change in balance between growth and fruiting associated with the increase in malt concentration from 0.01 to 0.1 g./100 ml. was not accompanied by a change of total fungus weight, although this rose steeply ($P = < 0.01$) with a further increase of malt concentration.

Only in the medium containing 1.0 g./100 ml. of malt extract was the final hydrogen-ion concentration (Table II) such as to inhibit primordium production (see experiments on hydrogen-ion concentration). It offers no solution of the changed proportions of mycelium and sporophore dry weights with an increase of malt from 0.01 to 0.1 g./100 ml. The enhanced total fungus weight when malt content was raised to 1.0 g./100 ml. was probably not due to the alkaline drift of the medium (see hydrogen-ion concentration experiments), and malt extract (2 g./100 ml.) used as a complete medium became more acid (Table II). Thus not a hydrogen-ion but another, perhaps nutritional, influence may be involved. A more complete consideration of this effect is deferred to the discussion. Further work is in hand.

The foregoing observations suggested that the synthetic medium has advantages for the study of fruit-body production although giving low mycelium yields.

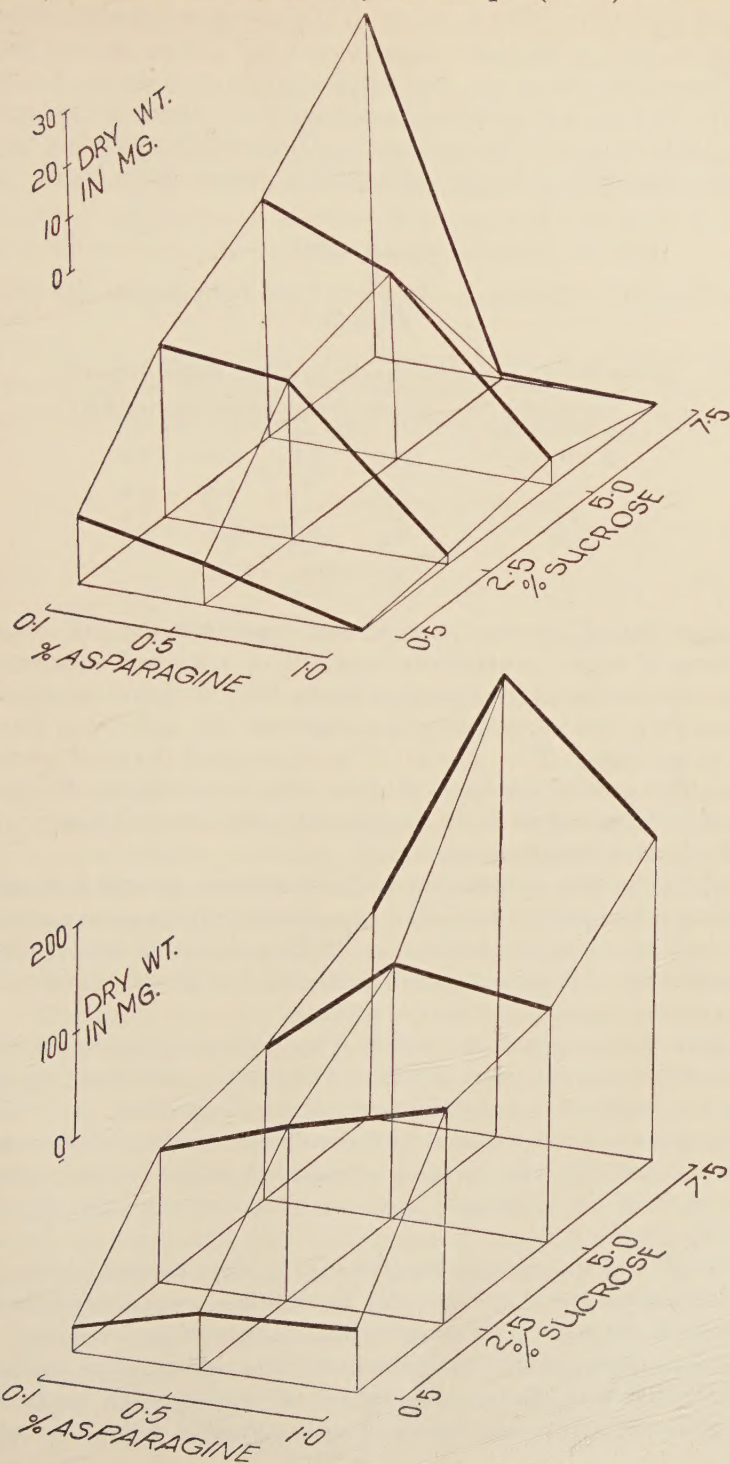
2. *The influence of the concentration of the carbon and nitrogen sources of the medium*

The effect of varying sucrose and asparagine concentrations simultaneously. In an attempt to determine the optimum concentrations of sucrose and asparagine for fructification a series of quantitative variants of the medium were prepared. These comprised sugar concentrations of 0.5, 2.5, 5.0, and 7.5 g./100 ml. at three asparagine levels: 0.1, 0.5, and 1.0 g./100 ml., the media being similar in their other components. Culture and harvesting (at 39 days) were effected in the usual way. Mean dry weights of fruit-body and mycelium crops are plotted in Text-fig. 1.

Lower asparagine concentrations were associated with greater sporophore yields. These were significantly higher with 0.5 g./100 ml. than with 1.0 g./100 ml. of asparagine ($P = < 0.01$) except at the sucrose level 7.5 g./100 ml. Even here, further reduction of asparagine concentration to 0.1 g./100 ml. resulted in a significant yield increase ($P = < 0.01$).

Over the range studied sugar concentration had a different effect. Except where the inhibitory action of high asparagine concentration (1.0 g./100 ml.) might be assumed to be limiting, increase of sucrose concentration from 0.5 to 5.0 g./100 ml. caused a significant yield increment ($P = < 0.01$). The highest mean yield of sporophores (sucrose 7.5, asparagine 0.1 g./100 ml.) was based on a very variable sample, and comparison with the yield at sucrose 5.0 g./100 ml. and the same asparagine level failed to establish a significant difference ($P = 0.3-0.2$).

Many factors besides the actual amounts of carbon and nitrogen available to the fungus may have been active in producing the spectrum of yields shown in Text-fig. 1. Media with high asparagine or low sucrose content were finally more alkaline (Table III) than the maximum limit for primordium production (about pH 7.0). It might be supposed that here, as in many mould fungi, the utilization of asparagine results in alkaline and of sucrose in acidic metabolic products, so that where the unbalance of the metabolites is greatest



TEXT-FIG. 1. Mean dry weights of sporophores (above) and mycelia (below) upon media of different initial sucrose and asparagine content. Incubation time 39 days. Replication 5 ×.

the largest drift in reaction is observed. High sucrose concentration delayed the onset of fruiting (see later experiments) and it may be that the lowest yields obtained (at sucrose 7.5, asparagine 0.5, and 1.0 g./100 ml.) were traceable to the reaction of the medium having reached a prohibitively high pH by the time that fruiting was due to commence. The possibility of staling substances having an effect independent of hydrogen-ion concentration is not ruled out.

TABLE III

The Final pH of Cultures on Media of Varied Asparagine and Sucrose Content

Initial pH 5.7. Incubation time 39 days. Replication 5 ×

Asparagine concentration (g./100 ml.)	Sucrose concentration (g./100 ml.)			
	0.5	2.5	5.0	7.5
0.1	7.0	*	6.4	6.4
0.5	8.0	7.4	7.1	7.4
1.0	8.4	8.0	8.0	8.3

* Not recorded.

Mycelium yields (Text-fig. 1) were in sharp contrast to those of fruit-bodies. The raising of sugar concentration resulted in a marked enhancement of mycelium production at all asparagine levels. Only at the initial sucrose concentration 7.5 g./100 ml. was growth significantly less with 1.0 g. than 0.5 g./100 ml. of asparagine ($P = < 0.01$). This represented the most concentrated medium. The greater tolerance of more alkaline conditions by growth as compared with fruiting no doubt contributed to the observed results, although other factors may have been operating.

Essentially the same response to media of different sucrose and asparagine concentration was noticed in similar experiments. At sucrose concentrations of 25 g./100 ml. or more, however, no fruiting occurred even at favourable asparagine levels. Mycelium growth occurred, but at a reduced level, in a medium containing 50 g./100 ml. of sucrose.

The effect of asparagine concentration. The influence of sucrose and asparagine concentrations were next considered separately, each being studied at a fixed and approximately optimal concentration of the other.

Firstly cultures were prepared with the sucrose content of the medium at 5 g./100 ml. Amounts of asparagine were added to give twelve replicate cultures at each of the asparagine concentrations 0.0, 0.05, 0.1, 0.5, and 1.0 g./100 ml.

Dry weights of sporophores harvested at 33 days confirmed the previous results in essential features (Table IV). In addition it was apparent that about 0.1 g./100 ml. of asparagine was approximately optimal for fruit-body production under the given conditions. Thus yields were significantly lower at 0.05 g./100 ml. ($P = < 0.01$) and at 0.5 g./100 ml. ($P = 0.05-0.02$). No fruit-bodies were produced on the medium lacking asparagine. Below the optimum,

asparagine concentration probably limits as a nitrogen source, while above the optimum the adverse influence already noticed becomes manifest. Mycelium yield showed significant increases with the rise of asparagine concentration from 0.05 to 0.5 ($P = 0.05-0.02$) and from 0.5 to 1.0 g./100 ml. ($P = < 0.01$), thus again showing a totally different response from fruiting. The greatest mycelium yield occurred in this instance at a higher asparagine level (1.0 g./100 ml.) than in the previous series at the same initial sucrose level. The different duration of the two experiments may be important here.

TABLE IV

The Effect of Initial Asparagine Concentration upon Mean Dry Weights (mg.) of Mycelium and Sporophore Crops and upon the Numbers of Cultures producing Fully Expanded Sporophores (Stage 3)

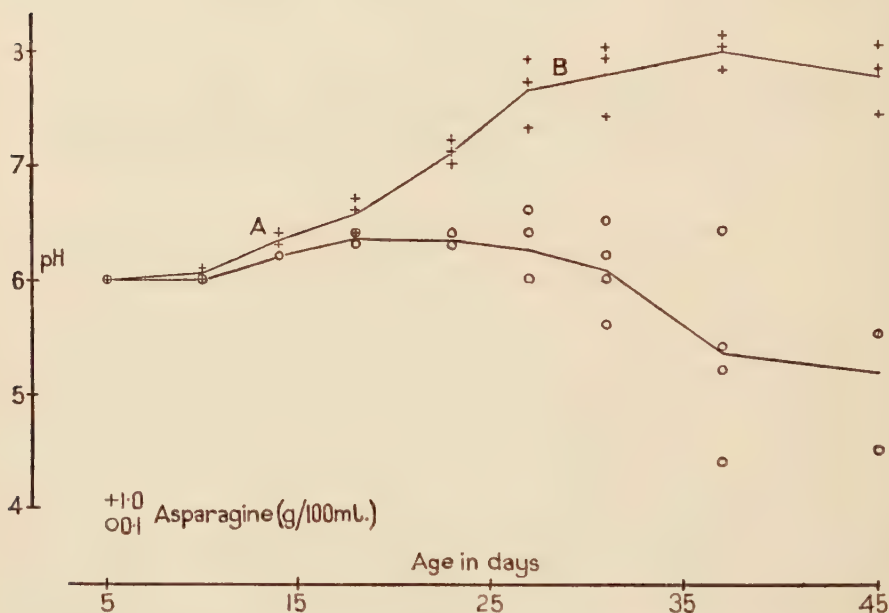
	Incubation time 33 days				
	Asparagine (g/100 ml.)				
	0	0.05	0.1	0.5	1.0
Sporophores	0 (12)	64.2±4.1	94.7±7.7	63.2±10.9	51.9±8.0
Mycelium	4.9±0.2	26.9±3.9	32.4±3.0	48.4±8.0	82.2±9.3
Cultures at stage 3	0*	12	12	5	0

* Not-fruiting.

Striking differences in the appearance of fruit-bodies were noticed. With 1.0, and to a less marked extent with 0.5 g./100 ml. of asparagine sporophore development, after starting normally, became slower and was finally arrested when most fruit-bodies were about half-grown. An intense darkening of the caps and stipes occurred (Pl. VI, Fig. 5). A concentration of 0.5 g./100 ml. of asparagine represented a critical level, as a few sporophores in this sample expanded completely while others were arrested before reaching that stage. When the experiment was repeated, daily records of the stage of development of cultures were kept (see 'Methods'). Rudiments (stage 1) became visible at about 14 days at all concentrations (contrast with sucrose concentration). With moderately high asparagine concentration (0.5 g./100 ml.) growth was apparently arrested or very slow, for a period of many days in either primordia (stage 1-2) or visibly differentiated sporophores (stage 2-3). Subsequently, a renewal of growth, detected by the longer duration of the experiment (50 days), resulted in some expanded fruit-bodies (stage 3) appearing in all cultures. Thus the adverse effect of this initial concentration of asparagine can to some extent be overcome. As before, with twice as much asparagine there was no renewal of growth and no expanded sporophores were produced.

The darkened appearance of fruit-bodies on the asparagine-rich media suggested that a staling principle might be involved. A comparison of the drift of hydrogen-ion concentration of cultures upon media with adverse

(1.0 g./100 ml.) and favourable (0.1 g./100 ml.) asparagine concentrations was in accord with this supposition (Text-fig. 2). Random samples of 3 or 4 from groups of 6 cultures were taken at intervals of a few days and their pH determined, using the methods described later (see experiments on hydrogen-ion concentration). A difference became apparent after about 10 days. At 20 days the richer medium showed a sharply rising pH, but the other little change from its initial reaction. Primordia appeared in the two groups almost



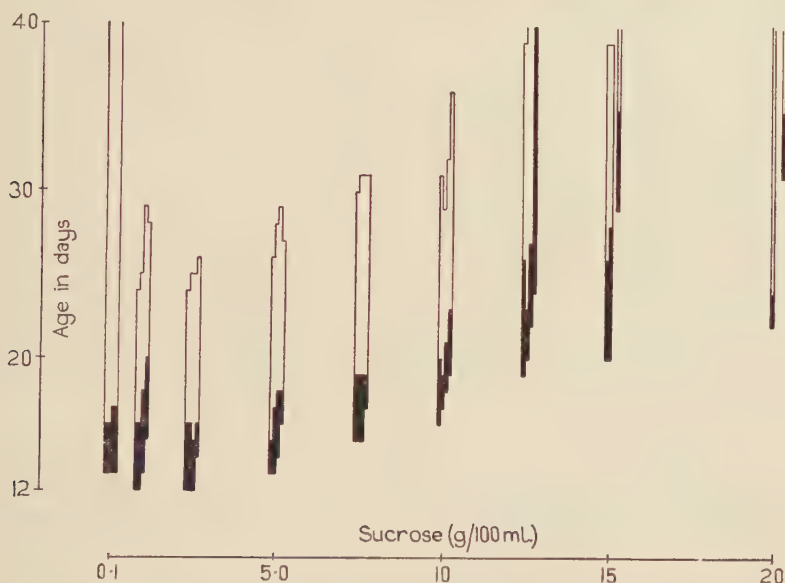
TEXT-FIG. 2. The drift of pH in media of different initial asparagine concentration. A: primordium production, B: cessation of sporophore growth.

simultaneously (point A on graph). At 27 days and a pH of 7.6 (point B on graph) it became obvious that sporophores in the cultures containing more asparagine had ceased growing, the actual stop no doubt occurring several days earlier. The final stage of the curve for the asparagine-rich medium shows a tendency to fall. Perhaps the cultures which exhibited renewed growth on media containing initially 0.5 g. asparagine/100 ml. in previous experiments were enabled to do so by a fall in pH.

Moist litmus-papers turned blue if suspended over cultures of high initial asparagine content, but not over media containing 0.1 g./100 ml. or less of asparagine. If a sterile air-stream was passed over the 1.0 g./100 ml. asparagine cultures and bubbled through water it gave a positive reaction with Nessler's reagent. The evidence points rather strongly, but not conclusively, to an ammonia staling as responsible for the reduced yields and stunted, darkened sporophores of asparagine-rich cultures. Further discussion is given later.

The effect of sucrose concentration. Further evidence of the influence of sucrose concentration was sought by adding to samples of the synthetic medium amounts sufficient to give a range of initial concentrations from 0.1 to 20.0 g./100 ml. Asparagine concentration was 0.1 g./100 ml.

A daily record of the course of development of sporophores on these media is presented in the diagram, Text-fig. 3. The important points illustrated are: firstly, that the characteristic period for complete development of a fruit-body,



TEXT-FIG. 3. The effect of different initial sucrose concentrations upon the times of fruiting-stages. At each concentration the history of each fruiting-culture in the sample (usually 4) is shown. The shaded portion = stage 1 to stage 2. Unshaded portion = stage 2 to stage 3 or the end of the experiment, whichever was the shorter. For description of stages see 'Methods' and Pl. V, Figs. 1, 2, 3.

which was not appreciably affected by sugar concentration, was approximately a fortnight. From stage 1 to stage 2 occupied 2-4 days. Some 8-12 days more elapsed before stage 3 was reached. This rather gradual development contrasts with the rapid growth of certain *Coprinus* sporophores (Borriss, 1934).

Secondly, the onset of fruiting was distinctly delayed by increased sucrose concentration. Primordia appeared at least 7 days earlier over media with an initial sucrose concentration of 1.0 g./100 ml. than over those with 12.5 or 15.0 g./100 ml. The later sporulation of fungi upon more concentrated media is not an unfamiliar occurrence. The longer period taken for the dilution of the medium, by fungus growth, to a level favouring reproduction is often advanced in explanation. Measurement of sugar depletion would be necessary to settle the matter.

Thirdly, not-fruiting cultures were in evidence at the highest concentrations, there being 1 out of 4 at 15 g./100 ml. and 2 out of 4 at 20 g./100 ml. sucrose.

It is highly likely that a relatively small further rise in sugar concentration would have resulted in the total elimination of fruiting. Such has actually been observed with 25 g./100 ml. of sucrose at only slightly higher asparagine concentration (0.25 g./100 ml.)

Fourthly, at very low sucrose concentration (0.1 g./100 ml.) sporophore growth ceased before maturation. Differentiation (stage 2-3) but no further growth occurred. Pl. VI, Fig. 6, shows such sporophores which should be compared with those in the cultures of the same age on 12.5 g./100 ml. sucrose (Pl. VI, Fig. 7). It is perhaps worthy of note that sugar starvation here results in numerous abortive sporophores rather than fewer more perfect ones.

All these features were present in a repetition of this experiment. Where primordium production is not synchronous it is clear that the relative magnitude of yields will depend on the time of harvesting. The most reliable measure of the capacity for sporophore production is presumably one made when their growth has ceased in all cultures. In practice harvesting was done earlier in order to avoid change of weight in the first-formed crops by autolysis or overgrowth by mycelium. When the yields were determined at 40 days fructifications were still expanding in two cultures at 20.0, one at 15.0, and three at 12.5 g./100 ml. sucrose (Text-fig. 3). These and not-fruited cultures were disregarded in calculating the mean sporophore yields (Table V and Text-fig. 4).

TABLE V

Numbers of Cultures with Mature Sporophore Crops and Mean Mycelium and Sporophore Yields (mg.) upon Media of Different Initial Sucrose Concentration.

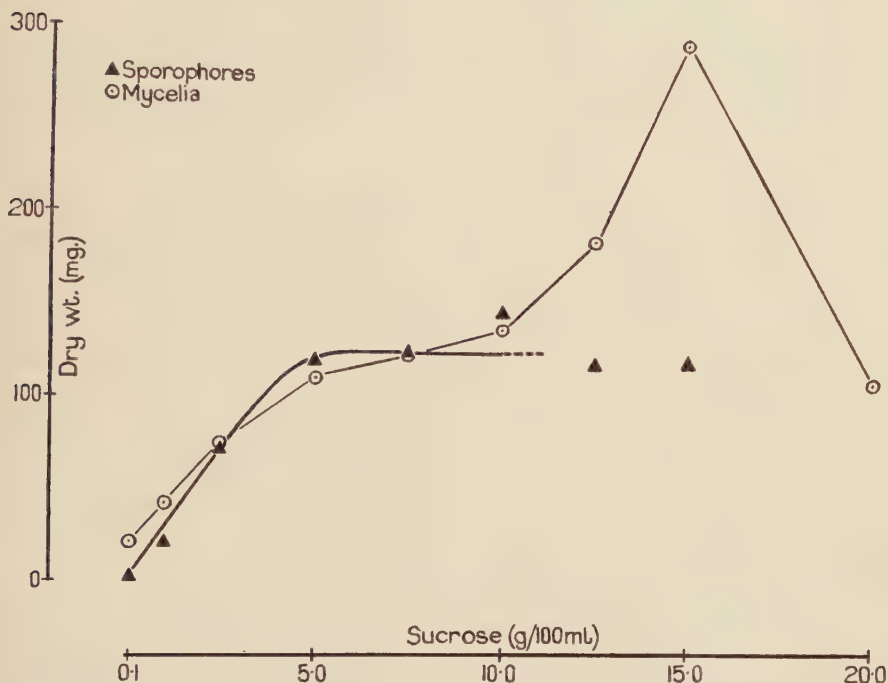
	Incubation time 40 days								
	Sucrose concentration (g./100 ml.)								
	0.1	1.0	2.5	5.0	7.5	10.0	12.5	15.0	20.0
No. of cultures with mature crops	0	4	4	4	4	4	1	2	0
Sporophores	2.5 ± 0.6	21 ± 1.6	71.5 ± 3.0	119 ± 14.2	123 ± 14.7	144.7 ± 22.4	117	118 ± 14	(92)*
Mycelia	20 ± 0.4	41.2 ± 3.7	73.5 ± 6.7	109.2 ± 9.6	121 ± 12.1	135.5 ± 6.5	182.5 ± 34.3	289.2 ± 36.0	106.5 ± 27.5

* The dry weight of a group of sporophores about three-quarters grown.

In confirmation of the previous observations, significant increases in mean yields of sporophores occurred for each rise in sugar concentration up to 5.0 g./100 ml. ($P = < 0.02$). Above that level a flattening-out occurred and differences ceased to be significant. At higher concentrations the limited data available suggest that, provided sporophore production starts, yield of fruit-bodies does not diminish. Even over a medium containing 20 g./100 ml. of sucrose a partially grown crop had a dry weight approaching those at lower concentrations (Table V). On the other hand, this and other experiments show that not-fruited cultures tend to occur in increasing numbers. It may well be that primordium production and final yield are independently determined

(see also pH data). If this is so, the conception of an optimum concentration for yield of sporophores is probably not justified. If, conversely, not-fruiting were regarded merely as zero yield and included in estimates of fruiting, then the curve would show a fall at values exceeding 12.5 g./100 ml. sucrose and an apparent optimum would emerge.

With the exception of the case mentioned (initial sucrose concentration 0.1 g./100 ml.) no arresting of fruit-body growth was noticed. Furthermore,



TEXT-FIG. 4. The effect of initial sucrose concentration upon mean dry weights (mg.) of sporophore and mycelium crops.

the final reaction of all media was not lower than pH 5.0 or higher than pH 6.4, so that hydrogen-ion concentration probably had little determinative significance in the trends observed. The failure of increases of initial sucrose concentration above 5 g./100 ml. to enhance sporophore yields is unlikely to be due to an insufficiency of other nutrients in the medium as mycelium growth continued to increase steeply above that concentration.

An optimum sucrose concentration for mycelium growth occurred, as mean yields were significantly higher at 15 g./100 ml. than at 20.0 or 10.0 g./100 ml. ($P = 0.05-0.02$).

That the contrasted responses of reproduction and growth to increase of initial sucrose concentration in the medium is due to some concomitant of concentration rather than amount of sucrose available as a nutrient is suggested by the experiment which follows.

3. *The effect of the volume of medium*

Cultures were prepared on 5.25-, 10.5-, 21.0-, 31.5-, and 42.0-ml. portions of the standard medium (sucrose 7.5, asparagine 0.1 g./100 ml.). Despite the form of the culture vessels (150-ml. conical flasks), the range in area of the medium-air interfaces was slight (about 28.3 to 31.1 cm²), due to the convexity of the base of the flask walls. Depths of medium varied from about 0.5 to 1.8 cm. Daily records of development were kept and mycelia and sporophores were harvested at 43 days, when reproductive activity had ceased.

The results (Table VI) were very striking. Fruiting started and was completed at approximately the same time in all cultures, except two of those on 5.25 ml. of medium where drying-out doubtless prevented the complete expansion of sporophores.

TABLE VI

Effect of Volume of Medium upon Dry Weights of Sporophore and Mycelium Crops (in mg.) and upon Numbers of Expanded Fruit-bodies

	Incubation time 43 days				
	Volume of Medium (in ml.)				
	5.25	10.5	21.0	31.5	42.0
Sporophores	16.2±8.8 (4)	53.7±8.0	143.0±23.6	254.2±45.4	300.7±28.2
Mycelia	150.7±38.0	102.7±16.1	109.5±22.4	115±20.4	139±35.7
No. of expanded sporophores	0.75±0.48	1.2±0.25	2.5±0.5	5.0±1.1	4.5±1.3

Significant increments of fruit-body yield occurred with a rise in volume from 5.25 to 10.5 ($P = 0.02-0.01$), 10.5 to 21.0 ml. ($P = 0.02-0.01$), and 21.0 to 42.0 ml. ($P = < 0.01$). Mycelium yields, on the other hand, did not vary significantly.

Close comparison between this experiment and the one concerned with sucrose concentration can be made, for they ran simultaneously under identical external conditions. The 3 days' difference in duration would be without effect upon sporophore yield because fruiting was completed. Thus the 21-ml. sample (volume experiment) was in all ways similar to the 7.5 g./100 ml. sample (concentration experiment), as is borne out by a comparison of their sporophore yields (143±23.6 and 123±14.7 mg. respectively). With a doubling of sucrose concentration up to 15 g./100 ml. (a) mycelium yield increased, (b) primordium production was delayed, and (c) sporophore yield did not increase, but with a doubling of volume up to 40 ml., which would similarly double the amount of sucrose, (a) mycelium yield did not increase, (b) sporophore production was not delayed, and (c) sporophore yield increased.

This comparison suggests that delay is associated with some aspect of concentration rather than the amount of sucrose available. Perhaps it is the

absence of delay in primordium production which allows sporophore production to proceed at the expense of growth when volume is increased.

The additional amounts of other nutrients than sucrose present in increased volumes of medium cannot be disregarded, but it seems unlikely that these limit yield in the higher sucrose concentration samples (see above).

Numbers of fully expanded sporophores were greater as volume of medium was increased (Table VI).

THE INFLUENCE OF HYDROGEN-ION CONCENTRATION

Two experiments were conducted to determine the sensitivity of the fruiting processes to the hydrogen-ion concentration of the medium. As the procedure in both cases was essentially the same they may be considered together.

Because of their possibly adverse influence buffer salts were not used in the medium (approximately N/20 K_2HPO_4 and K citrate buffering proved highly toxic in preliminary tests). The method of determining the drift of pH from different initial levels was adopted.

Samples of the synthetic medium (sucrose 7.5, asparagine 0.1 g./100 ml.) were adjusted to various reactions using normal HCl or KOH. In the alkaline range a precipitate (? magnesium phosphate) formed. This was not removed, but precautions were taken to ensure its proportionate distribution among the replicates concerned. The pH of the same two cultures of each sample was determined at intervals of a few days. The remaining six cultures of the group were harvested (at 32 days in expt. 1 and 43 days in expt. 2). Daily records of development were kept.

Determinations of pH were made with 'B.D.H.' capillator tubes and colour standards. The use of aseptic precautions in the removal of the tiny droplet of medium required for a determination made possible repeated estimations on the same cultures. This advantage outweighed the imprecision of the method (subject to a maximum error of about 0.3 of a pH unit). The highly exact values often published are meaningless when they relate to unshaken cultures. Throughout the experiments the tested cultures of a sample showed a closely similar reaction at any given time and may be presumed representative of the sample. Drift of pH in these cultures is shown in Text-fig. 5. It suggests that the natural buffering of the media was fairly good over the range tested.

Primordia appeared in the flasks of initial pH 5.0 between the 15th and 17th days when the tested cultures showed a reaction between 5.2 and 5.5. The last stages of stipe and cap expansion in these cultures occurred near the 30th day and coincided with a pH of 4.2 to 4.4. The association of these low values with active expansion is especially interesting because cultures of initial pH 4.5 (expt. 1) failed to fruit during a period of 32 days although their reactions exceeded pH 4.5 after about three-quarters of that time. It is evident that sporophore expansion can be completed at a lower pH than is necessary for primordium production.

The upper limit for fruiting was indicated by the cultures of initial pH 7.4 (expt. 2). Only two cultures out of eight in this sample showed reproductive

activity (Table VII). One produced a single primordium 4 days before harvesting (pH *c.* 7.0). The other fruited normally, beginning at 20 days, when the reaction of the parallel cultures used for determination of hydrogen-ion concentration was pH 7.2.

Thus the approximate limits for primordium production are pH 5.2 ± 0.3 to pH 7.2 ± 0.3 .

Mycelium growth showed a wider tolerance. In a medium varying from pH 3.8 to 3.6 only a few minute submerged pellets were formed in 32 days. Cultures with an initial pH of 4.5 started growth similarly, but gave rise to surface mycelium by the time the reaction had risen to pH 4.8. The upper limit for growth was not determined, but it was still luxurious in a medium of pH 8.6 which fell gradually to pH 7.8. About 10 days after inoculation a thick brownish pad of limited extent had formed on the medium of initial pH 5.0, but at higher values the mycelium was extensive, whiter, and more submerged. Shortly afterwards these differences disappeared as a surface-mat was established in all cultures.

While the two experiments were quite consistent in indicating the pH limits of sporophore production and growth, minor differences were evident in the crop yields obtained (Text-fig. 5 and Table VII).

Comparison of mean yields of sporophores on media of initial pH. 5.3 and 5.0 (expt. 1) showed a barely significant difference ($P = 0.05-0.02$). With a starting pH of 6.8 (expt. 2) yield of fruit-bodies was lower than with an initial pH of 6.0 or of 7.4, again with a low degree of significance ($P = 0.05-0.02$). Considering the experiments individually the remaining yields did not show differences which passed the significance test. Thus with two exceptions the mean sample-weights lend themselves to the tentative conclusion that, over the range in which fruiting occurs, there is no sharply optimal hydrogen-ion concentration. The solitary culture maturing fruit-bodies on a medium of initial pH 7.4 was especially interesting in showing a yield comparable in magnitude with that at other levels (comparison with the mean at initial pH 6.0 gives $P = 0.7-0.6$). Thus the influence of a slightly alkaline medium was to reduce the incidence of fruiting in the sample without diminishing the final dry weight of those sporophore crops produced. Here again the scant evidence points to the independent determination of primordium production and final yield.

No consistent differences occurred in the times at which fruiting started, but in expt. 1 most cultures with initial pH 5.0 and 6.8 were a couple of days later in producing primordia than the rest, and in expt. 2 a delay of 3 to 6 days was associated with the increase of initial pH from 5.0 to 6.8. These differences were probably due to the time taken to establish a surface mat from the submerged inoculum, particularly as the mycelium was initially more diffuse at the higher pH values. Perhaps the anomalous yields mentioned were associated with this factor.

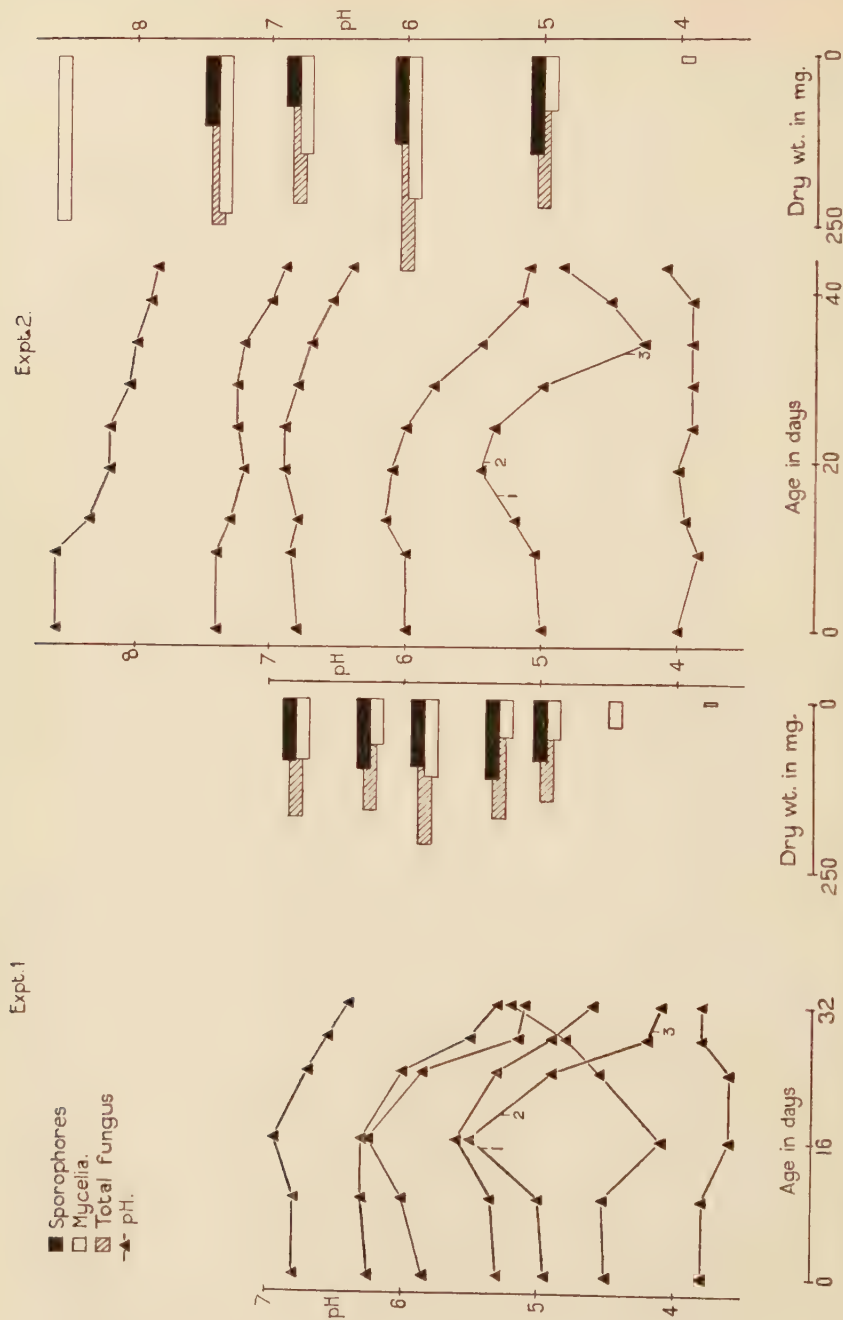
At the acid extreme an approximately linear response of mycelium yield to the rise in initial pH from 3.8-4.5-5.0-5.85 (expt. 1) ($P = < 0.01$) and from

TABLE VII

The Effect of Initial pH of Media upon Mean Dry Weights (mg.) of Sporophore and Mycelium Crops and upon Numbers of Cultures fruiting

Duration of experiments: expt. 1, 32 days; expt. 2, 43 days

Expt.	pH at inoculation	pH at inoculation									
		3.8	4.0	4.5	5.0	5.3	5.85	6.0	6.25	6.8	8.6
Expt. 1	Fruit-bodies	0	—	0	91±4.4	118±11.2	101±4.9	—	103±10.3	83±10.6	—
	Mycelia	3±0.4	—	39±12.7	57±3.8	58±5.9	115±14.4	—	64±3.1	91±8.2	—
Expt. 2	Fruit-bodies	—	0	—	144±14.5	—	—	107±21.7	—	74±3.7	0
	Mycelia	—	9±1.3	—	80±7.9	—	—	212±45.9	—	144±11.9	244±22.6
	No. of cultures fruiting	0	0	0	8	8	8	8	8	8	0



TEXT-FIG. 5. *Graphs:* pH drift from different initial levels in two experiments. 1, 2, 3: times at which the corresponding stages of sporophore development occurred. *Histograms:* Total, mycelium and sporophore mean dry weights (mg.) on media of different initial pH, in two experiments.

4.0-5.0-6.0 (expt. 2) ($P = 0.02-0.01$) occurred. The greatest mycelium yields in both experiments coincided with an initial pH value of the medium near 6.0. Above that level there was a drop in mean yield with the raising of initial pH level to 6.8 which was significant in expt. 1 ($P = < 0.01$) but not in expt. 2 ($P = 0.2-0.1$). Only in expt. 2 were more alkaline media considered, and here the mycelium yields did not differ significantly from those at pH 6.0. In these cases the absence of fruit-bodies in most cultures makes a comparison with total yields more meaningful. Thus in both experiments a moderately well defined optimum of total fungus dry weight was apparent in the region of initial pH 6.0.

CLUSTERING OF FRUIT-BODIES

Under the apparently unrelated conditions of suboptimal illumination and high asparagine concentration sporophores seemed to occur in clusters (i.e. the stipes were concrescent at the extreme base). Unfinished work suggests that high initial glucose content of the medium also has this effect.

Numerical expression was given to this phenomenon by calculating a coefficient of fasciculation (C.F.).

$$\text{C.F.} = \frac{\text{Total number of sporophores and primordia.}}{\text{Number of groups of sporophores and primordia.}}$$

This ratio was significantly higher for cultures in total darkness than in those receiving 6 or more hours of illumination per day ($P = 0.05-0.02$) (Pl. V, Fig. 4). Similarly samples on media of initial asparagine concentration 0.5 or 1.0 g./100 ml. gave strikingly higher values than cultures with 0.05 or 0.1 g./100 ml. ($P = < 0.01$) (Table VIII; Pl. VI, Fig. 5).

TABLE VIII

The Effect of Asparagine Concentration upon Mean Sporophore Numbers and the Coefficient of Fasciculation

	Asparagine concentrations (g./100 ml.)			
	0.05	0.1	0.5	1.0
Total sporophores (T)	14.1	16.2	27.8	25.5
Groups of sporophores (G)	7.2	7	6.6	6.3
T/G	2.0 ± 0.17 (12)	2.4 ± 0.18 (12)	4.7 ± 0.65 (10)	4.1 ± 0.44 (10)

In almost all cultures some primordia arose in such close proximity as to form what may be called 'compound primordia'. These may be the 'sclerotia' of Biffen (1898). Except in unilluminated cultures, some rudiments or even differentiated fruit-bodies usually abort. Thus, although increased fasciculation seems due to the larger numbers of rudiments in a compound primordium, this may be only apparent. Samples with little fasciculation are perhaps

those in which more rudiments abort at an early stage so that the most minute incepts are not recognized and counted.

The common factor operating in cultures with clustered fruit-bodies may be slow-growth conditions. With rapid expansion a few fruit-bodies possibly monopolize the available sporophore building materials, while slow growth and slower utilization of structural materials might enable more or all rudiments to continue development or more rudiments to be formed.

DISCUSSION

The dependence of pileus development in *Collybia velutipes* upon illumination is similar to that in such hymenomycetes as *Coprinus stercorarius*, *C. plicatilis* (Brefeld, 1877, 1889), *C. narcoticus* (Schenck, 1919), *Lentinus lepideus* (Buller, 1905), *L. tuber-regium* (Galleymore, 1949), *Schizophyllum commune* (Wakefield, 1909), *Polyporus squamosus* (Buller, 1906), and doubtless many others. That light is more than a triggering-force in this process is probably indicated by the increased amounts of sporophore material produced with longer daily periods of irradiation. With the use of higher intensities it is possible that the minimum period of light for normal development might be reduced as in *Coprinus lagopus* (Voderberg, 1950). Schenck's report (1919) that light intensity affected the relationship between stipe length and cap breadth in *C. narcoticus* is in general harmony with the result.

The very striking delay or inhibition of fruiting and increase in mycelium yield caused by adding yeast extract, certain samples of agar, and small amounts of malt extract to the synthetic medium requires further work for its explanation. Tentatively the effect may be regarded as not due to hydrogen-ion concentration and a nutritional action considered. Marczyński (1943) attributed the effect (upon mycelium growth only) to undetermined growth factors, because of the relatively low concentrations (yeast and malt extracts 0.02, 'bacto'-agar 0.4 g./100 ml.) required to cause a response in excess of the maximum thiamin effect. The hypothetical factors are not biotin, inositol, pyridoxine, or riboflavin (Barnett and Lilly, 1947a; Marczyński, 1943). Perhaps one or more amino-acids, which at low concentrations promote growth in many hymenomycetes (Melin and Norkrans, 1948; Norkrans, 1950; Fries, 1949), are the active agent(s). Glycine is inactive (Marczyński, 1943). The presence of available nitrogen in some agars is suggested by the report (Hawker, 1942) of growth and fruiting of *Collybia velutipes* on an agar medium with KNO_3 as the only added nitrogen source. The present strain did not utilize KNO_3 .

The small amounts of extracts active probably rule out a carbohydrate but not minerals or trace elements as the unknown agent(s). Further studies on the nitrogen and mineral nutrition of *C. velutipes* are desiderata. Keyworth (1941) notes that the intensity of fructification in cultures of *Coprinus ephemerus* was depressed if the small amount of lentil extract (0.05 g./100 ml.) added to supply growth factors was increased.

The presence of a volatile substance giving a positive Nessler's reaction associated with sharply increasing alkalinity in asparagine-rich cultures suggests that ammonia is responsible for the stunting and diminution of yield of sporophores. It remains to be established, however, that the Nessler-positive substance alone causes the high pH and whether hydrogen-ion concentration, or direct toxicity, or both, are responsible for the observed effects. Experiments on the influence of hydrogen-ion concentration failed to show abnormal fruit-bodies in cultures growing at the upper limit of the pH range for fructification so that direct toxicity is made more probable. Henry and Andersen (1948) have mentioned a possible toxic effect of ammonia apart from its influence on the hydrogen-ion concentration of cultures of *Piricularia oryzae*, but Salvin (1942) has shown that other staling principles may be acting in cultures of *Thraustotheca* sp. even when ammonia is being evolved. High concentrations of urea in culture media led to inhibition of fruiting or a thickening and stunting of sporophores of *Coprinus lagopus* in experiments of Voderberg's (1948). An ammoniacal smell was reported in these instances.

The interpretation here given to the influence of sucrose concentration upon fruit-body production hinges upon the assumption that the flattening of the curve at the 5.0 g./100 ml. level is not due to the limiting concentration of other nutrients in the medium. The reasons for this assumption are that mycelium and total fungus yields continue to increase steeply above this point, so that limiting substances would have to be requirements peculiar to the fruiting process. Nitrogen was presumably not limiting because asparagine increase did not raise the critical sucrose concentration (although the staling associated with higher asparagine concentrations is a complication). Thiamin was present in great excess of the usual physiological amounts, there being five times more than the quantity (107/100 ml.) found necessary by Hawker (1942) to give a glucose optimum for fruiting (estimated numerically) or not less than 5.0 g./100 ml. The foregoing caveat having been entered, the discussion may proceed.

In having a sugar concentration optimal for mycelium growth greater than the least concentration producing the maximum reproductive response, *Collybia velutipes* may perhaps be likened to such ascomycetes as *Pyronema confluens* growing on glucose or sucrose, or *Sordaria* (*Melanospora*) *destruens* on glucose media (Hawker and Chaudhuri, 1946). In these and other fungi, however, it is usual to find the inhibition of fruiting at high concentrations preceded at slightly lower concentrations by diminished yields (i.e. smaller numbers of perithecia, &c.). In *C. velutipes* no diminution in sporophore dry weight is in evidence until fruiting is inhibited entirely in some and finally all cultures. Thus there is no narrow optimum of initial sugar concentration for fruiting.

The advantage of dilute media for the early onset of sporulation is familiar in many fungi (Basu, 1952; Timnick *et al.*, 1952), but the causes remain obscure. A possibly higher respiration intensity of cultures on media of

lower sucrose content during the early stages of growth of *Collybia velutipes* (Plunkett, 1951) is of interest, as Hawker (1950, p. 154) has indicated that factors tending to increase respiration-rate in fungi may favour fruiting.

A very instructive comparison with the effect of sucrose concentration is provided by the response of the fungus to various volumes of a medium of initially favourable concentration. In *Sordaria fimicola* a later perithecial production with volume increase has been related convincingly to the sinking of the inoculum and retarding of surface mycelium formation (Barnett and Lilly, 1947*b*). No such delay occurred in this work, perhaps because the range of volumes and hence depths of medium studied was not great. The results point strongly to some concomitant of concentration rather than the amount of sugar available as determining the time of primordium production. Experiments in which the concentration factor is varied with non-nutrient substances should be applied to *C. velutipes*. It is tempting to suppose that the utterly different distributions of dry matter between fructifications and mycelia resulting from volume and concentration increases are related to the time of onset of fruiting.

In so far as they have been determined the cardinal points of hydrogen-ion concentration for growth in *C. velutipes* resemble those of many other white-rot fungi of timbers. A remarkable feature of the results was the failure of two experiments to demonstrate an optimum level for sporophore dry-weight production, although media of initial pH *c.* 6.0 bore the greatest total weight of fungus. This contrasts with Johnson and Jones's inadequately supported claim (1941) to have demonstrated coincident optima for fruiting and growth in *Coprinus ?cubensis*. The wide range of hydrogen-ion concentration for fruiting (pH 4.0 to 9.0) in *Coprinus lagopus* suggested by Voderberg's data (1949) similarly requires confirmation as pH drift was not measured and a very considerable delay in primordium production occurred in the acid media.

Fruiting in hymenomycetes is a complex process. In this work certain stages or aspects of the process have proved experimentally separable. Thus primordium production can occur in darkness, is limited to pH 5.2 to 7.2 approximately, and is inhibited by high (sugar) concentrations. Stipe elongation is also a dark reaction, but pileus expansion requires light. At least the final stages of both processes, however, can occur above more acid media (pH *c.* 4.2) than primordium production. These and probably other processes underlie the final dry weight of sporophore crops with which this paper is principally concerned.

Kleb's principle that the favouring conditions for reproduction are always more or less unfavourable to growth (1900) is not consistently borne out by results with *Collybia velutipes*. Increase of sucrose concentration, for example, may promote growth and fruiting equally over a considerable range. Steinberg's recent comment (1950) that 'the major bases for this belief' (in the opposed nature of reproduction and growth) 'are those cases in which supplies of metabolites are insufficient for both processes' is also not in accord with the experience that richer media often bear sterile and copious growths of

mycelium. Throughout, conditions for reproduction were clearly more restricted than those for growth.

SUMMARY

At 20° C. and with suitable illumination *Collybia velutipes* fruits freely upon a synthetic liquid culture medium of minerals, sucrose, asparagine, and vitamin B₁. Potassium nitrate is probably not utilized as a nitrogen source. The addition to the medium of various plant extracts (especially malt extract) is shown to enhance mycelium production but to diminish or suppress fruiting.

A low initial concentration of asparagine is optimal for fruit-body production. At higher concentrations sporophore crops of abnormal appearance and diminished dry weight are associated with marked alkalinity of the medium and the production of a volatile substance giving a positive Nessler's reaction.

Increase in initial concentration of sucrose up to a given level leads to an increased yield of sporophores. Higher concentrations delay the onset of fruiting and eventually reduce its incidence. Provided that primordia are produced, the final dry weights of sporophore crops are probably not diminished by high sucrose concentration.

The heaviest mycelial mats are formed in cultures with relatively high initial concentrations of sucrose and asparagine.

Over the range studied, increase in volume, without essential increase in the air interface of the medium, does not delay primordium production. It enhances fruit-body but not mycelium yields. By closely relating development to pH change in cultures of different initial reaction it is possible to show that mycelium growth occurs over the range of pH approximately 4.5 to more than 8.6. Primordia arise only when the medium has a pH of 5.2 to 7.2 approximately, although the final stages of sporophore development may be less limited in this respect. The results do not indicate a pH optimal for fruiting.

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DESCRIPTION OF PLATES

Illustrating B. Plunkett's article 'Nutritional and Other Aspects of Fruit-body Production in Pure Cultures of *Collybia velutipes* (Curt.) Fr.'

Scale lines on the photographs all represent 1 cm.

PLATE V

- 1-3. Stages in fruit-body development. 1. The primordium newly arisen upon the mycelium (stage 1). 2. Tiny stipes showing the beginning of pileus formation (stage 2). 3. An expanding sporophore in which the pileus has almost flattened (nearly stage 3).
4. Clustered fructifications with minute pilei, produced in cultures from which light was excluded.

PLATE VI

5. The effect of certain initial asparagine concentrations upon fruiting. Age of cultures 30 days. *Left-hand culture* 0.1 g./100 ml. asparagine. *Right-hand culture* 1.0 g./100 ml. asparagine showing clustered and deeply pigmented sporophores which have failed to expand.
- 6 and 7. The effect of certain initial sucrose concentrations upon fruiting. Age of cultures 39 days. 6. 0.1 g./100 ml. sucrose: differentiated but aborted sporophores. 7. 12.5 per cent. sucrose: normally developing sporophores.



1



2



3



4

B. E. PLUNKETT



Some Effects induced in *Nicotiana glutinosa* by the 'Aspermy' Virus of Tomato

BY

JOHN WILKINSON

(*University College, Exeter*)

With Plate VII

ABSTRACT

Inoculation of *Nicotiana glutinosa* with the virus causing 'aspermy' disease in tomato causes malformation of all parts, increased development of lateral branches, foliar and floral distortion, and reduced production of seed. Some anthers and ovaries become necrotic, but many undergo sporogenesis. At the first meiotic division the pachytene threads of some spore mother-cells exhibit collapse, accompanied by abnormal multiplication of nucleolar bodies; whilst pairing fails in one and sometimes two or three bivalents, resulting in a proportion of chromosome-deficient gametes, with consequent production of misshapen pollen-grains, together with microcytes.

INTRODUCTION

SINCE the discovery of a new virus disease of tomato, called 'aspermy', in this department (Blencowe and Caldwell, 1946), and the acquisition of more detailed information as to its host range and reactions (Blencowe and Caldwell, 1949), further avenues of inquiry have suggested themselves. The virus produces systemic symptoms in *Lycopersicon esculentum* and diminishes seed-production in the usually reduced and distorted fruits. Moreover, such seeds as may be produced are usually non-viable. One question of obvious interest concerns the possible effect of the virus upon sporogenesis, and a preliminary report of this in the tomato has been published (Caldwell, 1952).

Apart from the paper by Kostoff (1933), discussed by Caldwell (1952), the only observation of virus interference with nuclear behaviour is that of Sheffield (1941), who states that severe etch virus sometimes stimulates the nuclei of fully differentiated cells to divide, yielding binucleate cells. The writer has studied for the past 2 years the effect of the aspermy virus on *Nicotiana* species, with special attention to any apparent abnormalities of meiosis, and the following account deals with *Nicotiana glutinosa*.

TECHNIQUE

The seed of *N. glutinosa* came some years ago from Rothamsted Experimental Station, and stocks have been maintained by 'selfing'. The virus inoculum was obtained from infected leaves of Stonor's Moneymaker'

tomato by grinding with water and allowing to stand for $\frac{1}{2}$ -hour. Each 3-week-old seedling was inoculated by lightly stroking both surfaces of the four apical leaves with decanted sap containing powdered carborundum. Most plants thus treated during the height of the growing season developed marked symptoms of infection within a week. Thirty plants in each experiment, together with controls, were kept in a cool, insect-free greenhouse. Cultures set up in January, May, and early September gave substantially the same results.

Material for the study of chromosome behaviour was taken from the time the first buds appeared, about 14 weeks after germination. Acetocarmine smears of anther-contents were used for inspection, and permanent smears of material for critical study were fixed in one of the modified Navashin fluids and stained by Feulgen's method. A shortened iron-alum haematoxylin procedure gave the best preparations for photomicrographs. 'Squashes' of thick sections of ovaries provided sufficiently good temporary preparations for verification of some of the meiotic effects noted in examination of the pollen mother-cells.

OBSERVATIONS

Apical growth of all the infected plants was retarded, and the production of lateral branches increased (Pl. VII, Fig. 1). Infected plants usually formed stunted inflorescences at the normal flowering time, and approximately one-tenth of the plants then showed apical necrosis and died. Leaves from diseased plants were usually not more than half the size of comparable leaves on the controls. The larger leaves became progressively attenuated at the base. Leaves appearing later showed reduced lamina development and became almost filiform. The diseased leaves, which were darker green than normal, developed characteristically distorted blisters and, occasionally, enations. These were prominent and numerous in plants germinated in late August.

The inflorescences of the diseased plants were usually foreshortened, often markedly so. Some of the buds in almost all the diseased inflorescences developed chlorotic sepals, the essential organs became necrotic at an early stage of development, and the buds dropped before the corolla had emerged. Of those buds that continued to mature, many developed somewhat enlarged stamens and ovaries, accompanied by extreme reduction of the corolla. In some less seriously affected plants the corolla development was of normal size, but showed a deep splitting. In a few heavily affected plants which produced flowers the stamens and sometimes the ovaries developed small and distorted leafy outgrowths. One such atrophied stamen bore only half of an anther-loculus, the rest consisting of small distorted leafy outgrowths. This loculus, however, contained pollen grains, microcytes, and suspended meiotic stages. The usual first and second meiotic divisions apparently occurred in those stamens not arrested by necrosis, but the whole meiotic cycle apparently occupied several days, in contrast with that of the controls, in which all stages normally occurred on the same day. The prophase of the first division was

suspended for long periods, and stages of this together with first and second meiotic metaphases and anaphases, tetrads, and even mature pollen grains, were in several instances found in the same anther-loculus. Such a wide range of stages was never similarly encountered in the controls.

In the anthers of diseased plants many pollen mother-cells did not progress beyond the prophase of the first meiotic division. The pachytene threads collapsed (Fig. 2), and the amount of nucleolar substance increased. Frequently in these arrested cells two or three large nucleoli were visible at pachytene, with two to five small ones; in those pollen mother-cells which did develop further, as with the corresponding cells of the controls, the nucleolar complement appeared to consist of only one large nucleolar body. This accords with the possession by *Nicotiana glutinosa* of but one pair of satellited chromosomes in the somatic complement. The prophase collapse and apparent increase of nucleolar substance has also been noted in ovule 'squashes'. Faulty fixation was not responsible for the effect, as controls which had received identical treatment did not exhibit such collapse.

The most striking abnormality observed at the metaphase of the first meiotic division was a failure of pairing between the homologues of one, or sometimes more than one, bivalent. The unpaired chromosomes could often be seen in side views of the first meiotic spindle (Pl. VII, Fig. 3), and usually two or three such were detected at different levels of focus. As the unpaired univalents sometimes failed to travel to the poles with the other chromosomes, counts of polar metaphases at the first and second meiotic divisions occasionally revealed 11, 10, or 9 chromosomes instead of the normal 12. In more than 200 such counts 16 per cent. lacked one chromosome, whilst 7 per cent. lacked two and 3 per cent. lacked three. Thus about one-quarter of the pollen output of diseased plants might be expected to possess chromosome-deficient nuclei.

In the loculi of mature anthers of diseased plants were many misshapen pollen grains accompanied by microcytes (Pl. VII, Fig. 4).

The largest capsules formed by the diseased plants after self-fertilization were not more than two-thirds the normal length, and showed an average of 925 seeds per capsule, compared with the normal 1,245. Together with apparently normal seeds, the diseased capsules contained a dust which was probably the remains of undeveloped or aborted ovules.

DISCUSSION

So far as the writer is aware, this is the first record of aberrations induced in a complete meiotic sequence by the action of a virus. 'Aspermy' virus is somewhat drastic in its general effect upon *N. glutinosa* (and, incidentally, as noted in experiments now proceeding, upon *N. rustica*); and, on the other hand, various species of *Nicotiana* are known to be susceptible to the influence of external agencies. Thus *N. tabacum* exhibited meiotic abnormalities when irradiated by X-rays (Goodspeed, 1929), and abnormal progeny arising as the result of X-irradiation and also of radium emanations have been analysed

(Goodspeed and Avery, 1930). These meiotic abnormalities included non-conjunction, non-disjunction, fragmentation, and various types of structural reorganization. Indeed, X-irradiation has for many years been a recognized method of establishing, by design, monosomic strains of some *Nicotiana* species and those of other genera.

Whether the monosomic condition may be transmitted via virus-induced chromosome-deficient gametes is still under investigation. So far, the seeds produced in the capsules of diseased plants, unlike those of tomato infected with 'aspermy', have germinated well and produced normal seedlings. Probably the 25 per cent. of ovules which fail to function in the diseased capsules are those in which the gametes arose as a result of meiotic abnormality. Any $n-1$ microspores produced would almost certainly be non-effective because of certation. It is therefore probably not without significance that the proportion of abnormal microspores is roughly the same as that of the ovules which fail to develop into seed.

The floral and vegetative modifications induced by the virus resemble the aberrations exhibited by the progeny of X-irradiated *N. tabacum* (Goodspeed and Avery, 1934). The markedly reduced, split, and very asymmetrical corollas of diseased plants of *N. glutinosa* appear to have been paralleled in *N. tabacum* by X-irradiation and radium emanation. It is, indeed, a matter of general interest that so many cytogenetic variations in plants are so remarkably similar to types arising from virus disease, and possibly other mutations ascribed to cytogenetic aberration may be the result of virus infection. Chevalier (1949) states that many of the *Oenothera* and *Cymbalaria* mutations, especially those exhibiting marked floral aberration, are the result of virus disease. It may well prove to be the case that after further investigation some unexplained abnormalities in, for instance, *Nicotiana* or *Lycopersicon* species are induced by virus.

ACKNOWLEDGEMENTS

It is a pleasure to thank my colleagues, Professor J. Caldwell and Dr. G. C. Ainsworth for their many valuable suggestions and interest in the work.

DESCRIPTION OF PLATE VII

Illustrating John Wilkinson's paper on 'Some Effects induced in *Nicotiana glutinosa* by the "Aspermy" Virus of Tomato'.

- FIG. 1. Healthy control (left) and diseased plant (right) of *Nicotiana glutinosa*, aged 4 months.
FIG. 2. Collapsed threads at pachytene in pollen mother-cells of a diseased plant ($\times 1,500$).
FIG. 3. Unpaired univalent on spindle of meiocyte at metaphase of first meiotic division in anther from a diseased plant ($\times 1,500$).
FIG. 4. Pollen-grains and microcytes from anther of a diseased plant ($\times 1,000$).

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FIG. 1

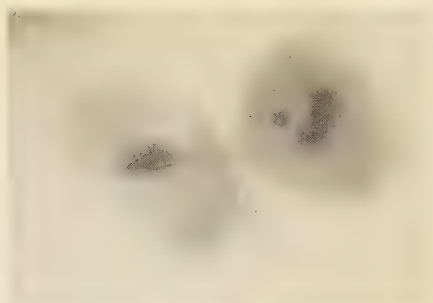


FIG. 3

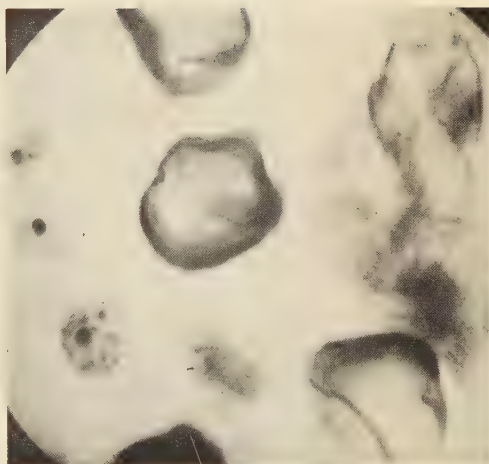


FIG. 4

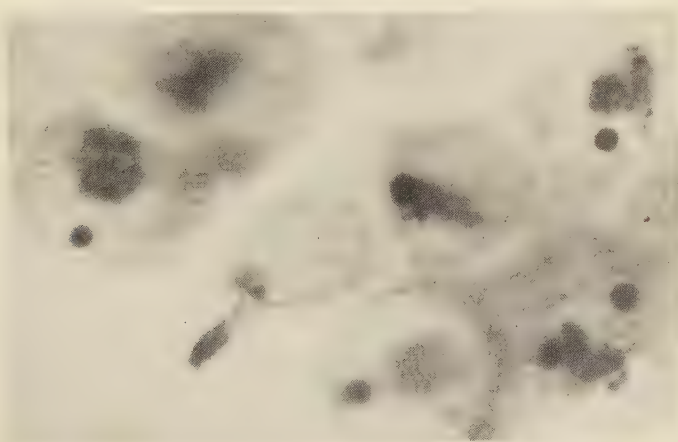


FIG. 2

Physiological Studies in Plant Nutrition

XVI. The Mineral Nutrition of Bracken

PART II. *The Effects of Phosphorus and Potassium Supply on Total Dry Weights, Leaf Areas, Net Assimilation Rates, Starch and Water Contents, &c., in the Sporophyte*¹

BY

W. W. SCHWABE

(Research Institute of Plant Physiology, Imperial College of Science and Technology, London)

With six Figures in the Text

ABSTRACT

1. The nutritional requirements of the bracken sporophyte were examined in a factorial combination of 3 potassium levels $\times$ 2 phosphorus levels $\times$ 3 solution types, in which the cations were mainly Na, Ca, or NH_4 .

2. The effects of nitrogen and phosphorus deficiency and of shading under conditions of high and low potassium supply were also examined.

3. Leaf area, total dry weight, and net assimilation rates are much depressed by lack of either K or P.

4. Water contents of leaves and rhizomes are generally increased by lack of K when Na is present in the culture medium but not when Ca is in excess. Phosphorus causes diminished succulence.

5. Starch content increases as phosphorus supply is lowered. With increasing doses of K, starch content falls in the high calcium solution, while increasing in the other two solution types.

6. Reduction of light intensity is shown to have a beneficial effect under conditions of K deficiency.

7. Analysis reveals complex interactions between the various factors, and possible toxic effects of Na, NH_4 , and excess P. An estimate of the relative importance of net assimilation rate, leaf number and area per leaf in determining total plant size has been calculated for the different nutrient treatments. Comparisons are made with previous results for barley and flax.

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¹ Part of thesis submitted to the University of London for the Ph.D. degree in 1949. This investigation was supported by a grant from the Agricultural Research Council.

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INTRODUCTION

THE first part of this paper (Schwabe, 1951) described certain experiments on the nutrition of the prothallial generation of bracken and the cultural techniques employed therein. The design of a large experiment on the mineral nutrition of the sporophyte generation was discussed and some of the results presented. These included leaf production and death-rates and numbers of leaf and rhizome buds in the different nutritional treatments.

The plants were grown at two levels of phosphorus supply (standard and $\frac{1}{10}$ th standard), and three levels of potassium supply (standard, $\frac{1}{3}$ th and $\frac{1}{81}$ st standard). Three basal nutrient media were used, the anions being supplied mainly as sodium, calcium, or ammonium salts. Four samples at fortnightly intervals were collected and also a final harvest (with twice the number of replicates) 6 weeks after the last sample. For convenience the table giving details of the nutritional treatments is repeated here (Table I). At the foot of this table are shown the symbols describing the various treatments.

TABLE I
Nutritional Scheme. Salts in g. per Pot

Salts.	Solution A.		Solution C.		Solution M.	
	HA.	LA.	HC.	LC.	HM.	LM.
NaNO ₃	9.11	9.11	—	—	—	—
NaH ₂ PO ₄ ·H ₂ O	1.098	0.110	—	—	—	—
Na ₂ HPO ₄ ·12H ₂ O	0.15	0.015	—	—	—	—
Na ₂ SO ₄ ·10H ₂ O	—	1.274	—	—	—	—
Ca(NO ₃) ₂	—	—	8.79	8.79	—	—
CaH ₄ (PO ₄) ₂ ·H ₂ O	—	—	1.06	0.106	—	—
CaCl ₂ ·6H ₂ O	0.37	0.37	0.37	1.199	0.37	0.37
MgSO ₄ ·7H ₂ O	1.25	1.25	1.25	1.25	1.25	1.25
NH ₄ NO ₃	—	—	—	—	3.928	4.246
(NH ₄) ₂ HPO ₄	—	—	—	—	0.918	0.092
(NH ₄) ₂ HPO ₄	—	—	—	—	0.055	0.0055

Potassium levels.
(g. per pot.)

K₁ = 1.85

K₃ = 0.206

K₅ = 0.023

Minor elements per pot.
(All pots.)

H₃BO₃ . . . 0.0286

MnSO₄·4H₂O . . . 0.0204

TABLE I—continued

Nomenclature employed

	Sodium soln.		Calcium soln.		Ammonium soln.	
	High phos.	Low phos.	High phos.	Low phos.	High phos.	Low phos.
High potassium . . .	HAK ₁	LAK ₁	HCK ₁	LCK ₁	HMK ₁	LMK ₁
Medium potassium . .	HAK ₃	LAK ₃	HCK ₃	LCK ₃	HMK ₃	LMK ₃
Low potassium . . .	HAK ₅	LAK ₅	HCK ₅	LCK ₅	HMK ₅	LMK ₅

To these the following should be added: S = age and S₁ to S₄ = sample number, F.H. = Final Harvest. Suffixes in brackets given in the tables below indicate statistically significant difference values appropriate to the particular part of the table.

RESULTS

Total dry weights. As the statistical analysis of total dry-weight yields per plant needed to be carried out on the logarithms of the original weights, the results also are presented in this form, except that ratios of actual treatment values are sometimes cited in the text. The final stage of the analysis is given in Table II, from which, however, values for first-order block interactions have been omitted. Variance ratios are shown for all relevant comparisons. The results grouped in Table III and shown graphically in Fig. 1 are presented as the mean log. per plant. It will be seen from Table II that age is the only main effect significantly greater than each of its interactions with other factors, indicating the general increase in dry weight, regardless of treatment.

Of the first-order interactions, that of P and K exceeds all others in importance (Table III (*b*); Fig. 1 (*a*)). Deficiency of either phosphorus or potassium alone strongly depresses total dry weight. Comparing the ratios of high and low phosphorus level yields (H/L) at the three potassium levels it is seen that a large effect is found only at the K₁ level. At the medium level the ratio is much reduced, while in the K₅ series an appreciable effect of phosphorus is lacking altogether.

	K ₁	K ₃	K ₅
H/L	6.1	1.9	1.1

The interaction diagram (Richards, 1941*a*) Fig. 1 (*a*) reveals that the gradient of the potassium-yield lines differs considerably at the H and L levels. When phosphorus is in short supply, the raising of the K level from K₃ to K₁ has less relative effect than raising it from the lowest level K₅ to K₃; the inverse holds at the high phosphorus level, where, moreover, the potassium effect between K₅ and K₃ is also much greater than at the low phosphorus level.

It is also of interest to compare the dry weights of plants grown at $\frac{1}{5}$ th the standard dose of potassium with those at $\frac{1}{10}$ th the standard level of phosphorus, a comparison which serves to indicate the relative importance of these two elements in the nutrition of the bracken sporophyte; the ratio HK₃/LK₁ is 1.5, and it is clear that under the conditions of the experiment reduction of

TABLE II
Logarithms of Total Dry Weight (decigrams)

	D.F.	Variance.	Tested against.	Variance ratio.	Tested against.	V.R.	Tested against.	V.R.	Tested against.	V.R.
Total	301	—	—	—	—	—	—	—	—	—
Blocks	.	.	Remainder	4.35*	—	—	—	—	—	—
P	2	0.1264	R.	387.2†	P × S	73.25†	—	—	—	—
K	1	11.2677	R.	467.2†	K × S	40.06†	—	19.53*	P × K	2.98
Soln.	2	13.5944	R.	57.0†	Soln. × S	10.87†	—	54.27†	P × K	3.59
S (Age)	2	1.6573	R.	228.0†	K × S	19.55†	—	6.62§	P × Soln.	2.87
P × K	4	6.6339	R.	130.0†	K × P × S	39.98†	—	43.50†	P × S	43.12†
P × Soln.	2	3.7829	R.	19.84†	—	—	—	—	—	—
K × Soln.	2	0.5770	R.	8.62†	—	—	—	—	—	—
P × S	4	0.2505	R.	5.29†	K × P × S	1.63	—	—	—	—
Soln. × S	4	0.1538	R.	5.25†	—	—	—	—	—	—
K × S	8	0.1525	R.	11.66†	K × P × S	3.59*	—	—	—	—
Soln. × P × K	8	0.3393	R.	1.71	—	—	—	—	—	—
Soln. × P × S	4	0.0497	R.	—	—	—	—	—	—	—
K × P × S	8	0.0267	R.	3.25†	—	—	—	—	—	—
K × Soln. × S	8	0.0946	R.	—	—	—	—	—	—	—
K × Soln. × S	16	0.0286	R.	—	—	—	—	—	—	—
Soln. × K × P × S	16	0.0244	R.	—	—	—	—	—	—	—
Remainder	.	.	.	.	.	.	.	.	.	.
	210	0.0291								

Significance level: * = 5 %; † = 1 %; ‡ = 0.1 %; § 5 % = 6.94.

phosphorus level is much more serious than reduction of potassium to practically the same extent. Although the interaction of these two factors with solution type does not reach significance level ($5\% = 2.26$), it may be noted that the ratio between the HK₃ and LK₁ treatments in the A solution is only 1.06, being therefore much lower than the corresponding ratios in the C and M solutions.

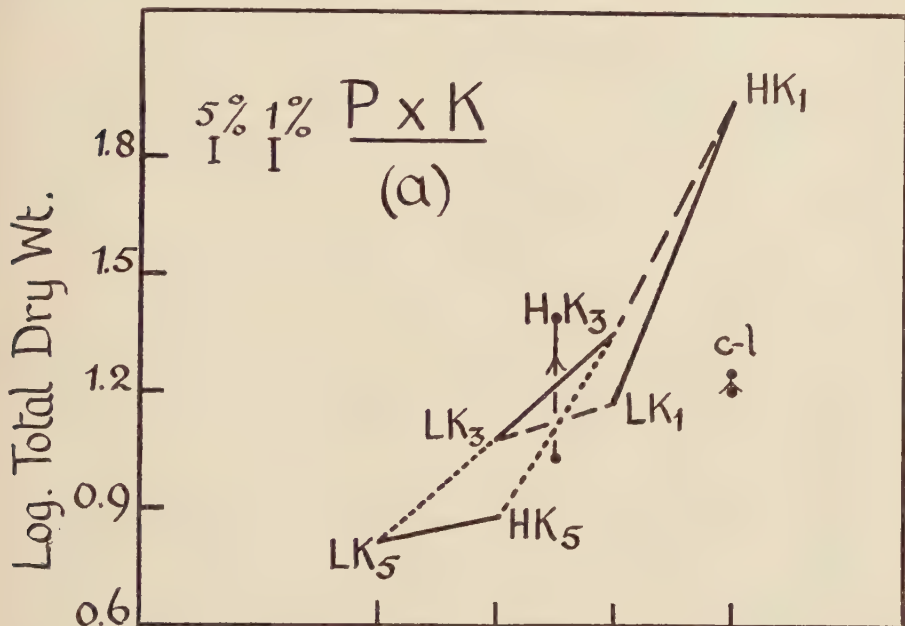


FIG. 1 (a). Interaction between the effects of phosphorus and potassium supply on log. total dry weight (the length of the central vector indicates the magnitude of the linear term of the interaction, the second vector labelled *c-l* the magnitude of the curvilinear term; both are positive).

The interaction of phosphorus level and solution type (Table III (c)) is also highly significant. According to the size of plant the three solutions fall into the following order at the two phosphorus levels:

$$H = C > M > A \quad L = C > A > M,$$

the high calcium solution being consistently the best; the order of the other two depends (significantly so) on phosphorus level. The ratios of the yields at the two phosphorus levels in the three solutions are:

	A	C	M
H/L	1.7	2.3	3.3

The interaction consists in the very different responses to phosphorus of plants in the A and M solutions. In the high sodium series this response is relatively small, a fact which is probably correlated with a faster rate of P absorption from this solution. The response to increased P dosage is particularly

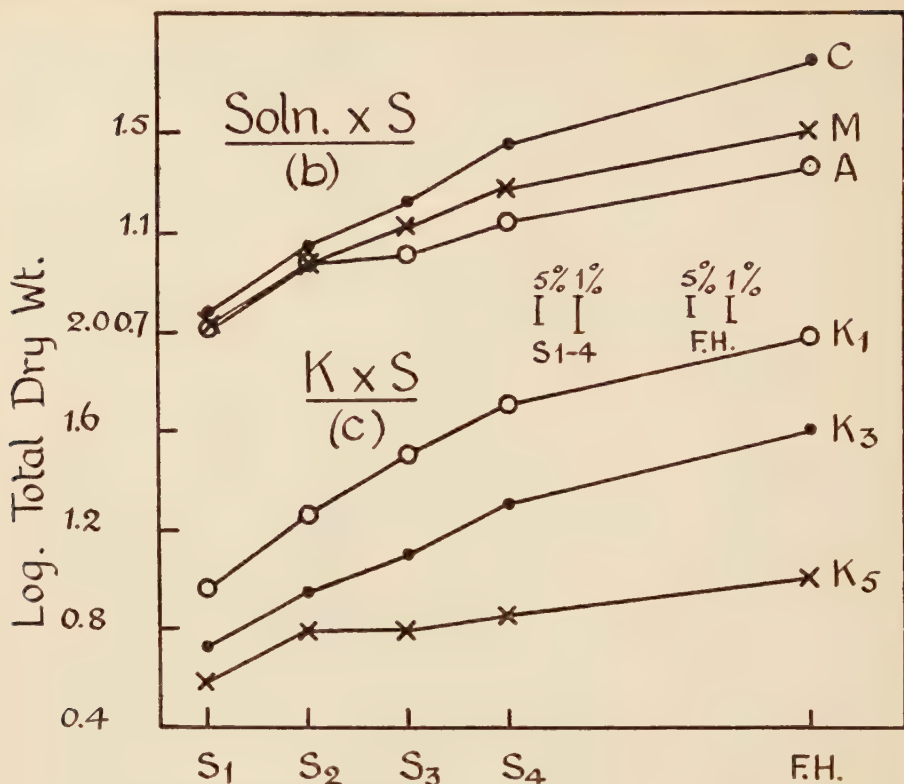


FIG. 1 (b) and (c). Effects of solution type and potassium level on log. total dry weight at different ages of plant.

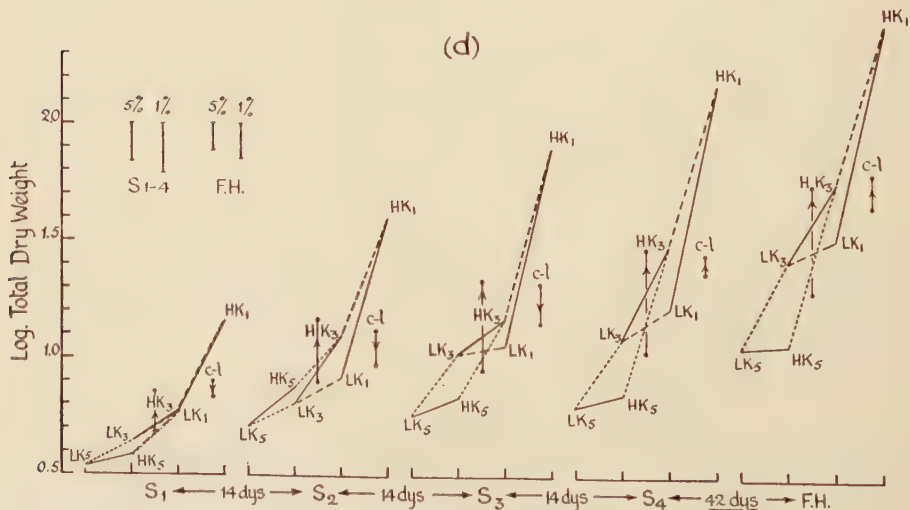


FIG. 1 (d). Interaction between the effects of phosphorus and potassium supply on log. total dry weight with increasing age of plant.

large in the M series. NH_4 toxicity found at the low level of P is regarded as the primary cause.

TABLE III
Logarithms of Total Dry Weights (decigrams)

(a) Age		S ₁	S ₂	S ₃	S ₄	F.H.
		0.749 ²	1.003	1.129	1.273	1.456 ¹
		H		L		
(b)	K ₁	.	.	1.956 ²	1.168	
	K ₃	.	.	1.345	1.073	
	K ₅	.	.	0.882	0.823	
		H		L		
(c)	A	.	.	1.218 ²	0.985	
	C	.	.	1.524	1.164	
	M	.	.	1.440	0.915	
		K ₁		K ₃	K ₅	
(d)	A	.	.	1.483 ³	1.142	0.679
	C	.	.	1.613	1.337	1.082
	M	.	.	1.590	1.147	0.797
(e) Significant differences		.P =		0.05	0.01	0.001
¹	Means of 108 replicates	.	.	0.045	0.060	0.076
²	" " 54 "	.	.	0.064	0.084	0.108
³	" " 36 "	.	.	0.079	0.103	0.132

Changes in total dry weight due to potassium supply also differ largely and significantly according to the relative proportions of the other cations present in the basal nutrient medium (Table III (d)). Dry-weight increases in all solutions are far from being proportional to potassium supply over the whole range, but the response in the sodium and ammonium type solutions is almost twice as great as in the C solution.

	A.	C.	M.
Linear term K_1/K_5	6.3	3.4	6.2
Curvilinear term $\text{K}_1 \times \text{K}_5/\text{K}_3^2$	0.76	1.05	1.24

Since yield even at the K_1 level is highest in the calcium solution, the interaction must derive very largely from the favourable effects of this solution under conditions of potassium stringency.

Solution type produces a highly significant interaction with age of plant which is almost exclusively due to the differences in the relative growth rates in the C and A solutions, the former being above, the latter below the average. These differences between the two solutions are manifest only after the second sample. The M solution closely approximates to the mean throughout the growing season (Fig. 1 (b)).

The relative rate of dry-weight increase tends to fall with age at all three potassium levels, but the initial rates and the times over which they are maintained of course depend on potassium level (Fig. 1 (c)). At the K₅ level the relative increase in the fortnight between the first and second samples is almost half that of the whole growing season, at the end of which the plants attain only three times their dry weight at the first sample. With $\frac{1}{3}$ th the standard quantity of potassium the rate of increase drops more slowly, the final dry weight being 7.6 times that at the first sample. When adequate potassium is present the rates of increase in the successive intersampling periods are as follows: $\times 2.0$, $\times 1.7$, $\times 1.6$, and for the last 6 weeks $\times 1.9$ (or $\times 1.24$ per fortnight), the final weight being 10.4 times the dry weight of sample 1. This interaction is significantly greater than that involving phosphorus supply in addition, and is therefore valid for both the H and L series.

The interaction between K and P is modified with age so that the $K \times P \times S$ interaction is highly significant. The form of the interaction does not change very much, but quantitatively it becomes much greater from sample 3 onwards (Fig. 1 (d)).

Dry-weight distribution. The percentage distribution of total dry matter has been calculated for three fractions: leaf matter, rhizome, and roots. The leaf matter itself comprises three fractions, whose distribution is shown diagrammatically in Fig. 2, together with the three main fractions for samples 1 and 3 and for the Final Harvest. Separate statistical analyses were carried out on each of the three main fractions of total dry weight, but being interdependent they will be considered here together.

In order to ensure a more normal error distribution than that of the original percentage figures the angular transformation was employed for the statistical analyses (Cochran, 1938). This transformation, as others of a similar kind, suffers from the disadvantage that the interpretation of data (especially of interactions) becomes very difficult, since it is almost impossible to envisage the meaning of comparisons between such transformed values. Fortunately, in the present case the percentage fractions of dry weight, particularly of leaf and rhizome, have values which lie in the middle region and consequently differences between original values and transformed figures are comparatively small. Hence it has been deemed permissible to present the original percentage in Fig. 2, and to interpret the data with regard to the more marked effects as if no transformation had been utilized in their assessment.

The effect of age is highly significant in all three analyses. The rhizome fraction increases steadily as the plant becomes older, the increase being at the expense of both leaf and root fractions. In the early stages this increase of the rhizome fraction clearly represents the production of new tissues, but later in the season (at the latest by the third sample) a considerable portion of the increase represents storage of carbohydrate—mainly in the form of starch (cf. below).

The age effect shows significant interactions with potassium level in the rhizome-fraction analysis and with solution type in the root fraction. The

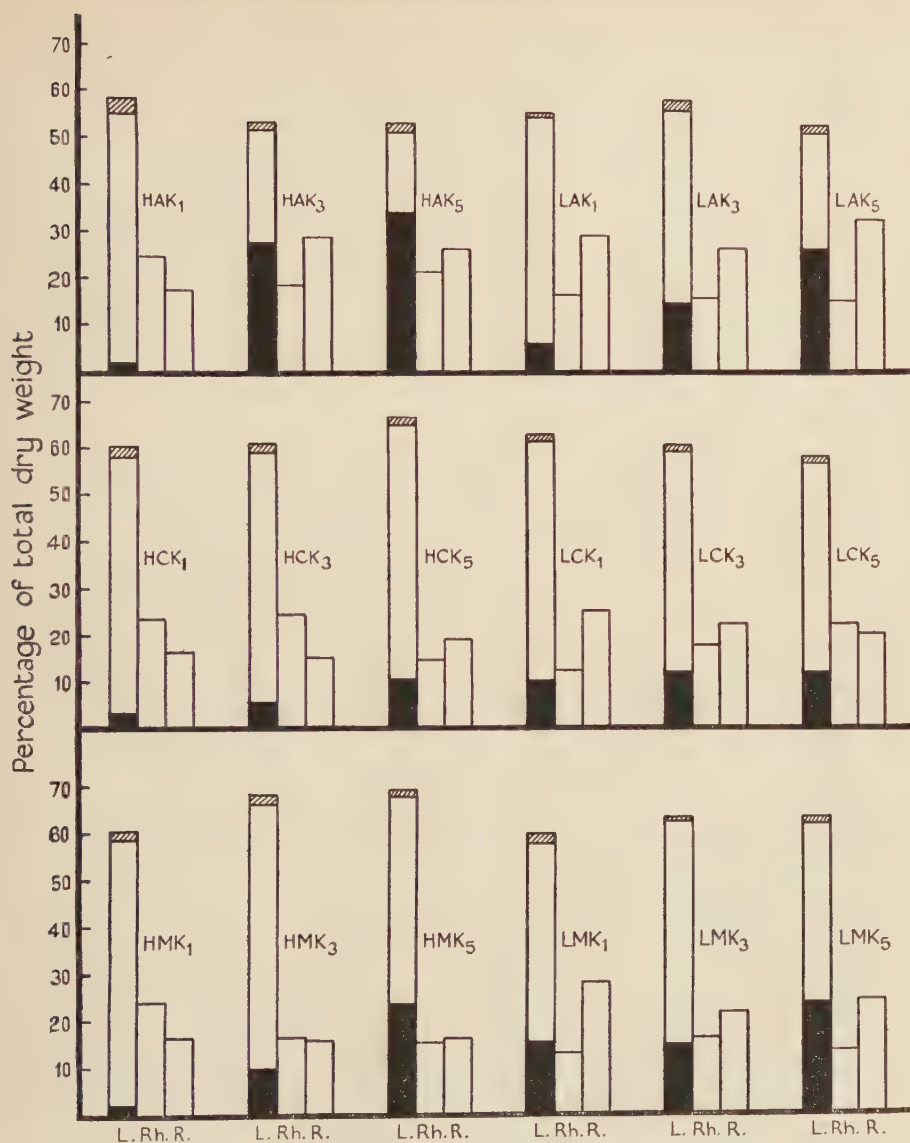


FIG. 2 (a) Total dry-weight distribution: first sample. The three columns for each treatment are Left to Right: Leaf matter (L.), rhizome (Rh.), and roots (R.). The total leaf matter is the sum of dead leaves (black), living leaves (white), and leaf buds and petiole bases (shaded).

rise in the percentage rhizome matter throughout the season proceeds at rates dependent on the potassium level. It is slower if potassium is in minimum supply and the final value reached is much below that of the K₁ series. The fall in the root fraction with increasing age is quickest in the A series during the first few sampling periods, values in the C and M types being almost

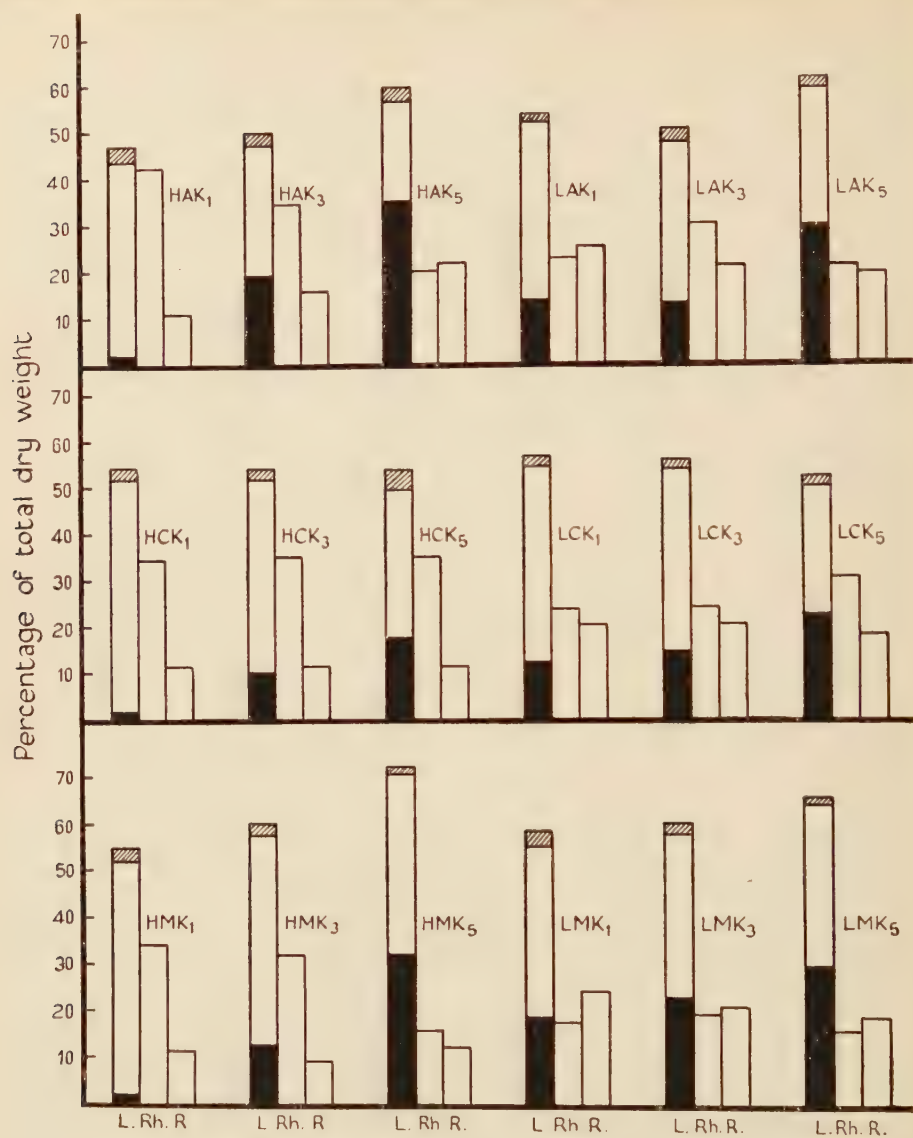


FIG. 2 (b). Total dry-weight distribution: third sample.

identical during this time, but later the C solution has relatively the least amount of root matter.

The main effect of solution type is significant compared with all significant interactions in which it is involved. Only in the analysis of the leaf fraction does solution type fail by a small margin to reach the conventional 5 per cent. significance level above all its interactions; hence one is justified in assuming the effect to be reasonably general. The three solutions, then, can be placed in the following order for each of the three dry-weight portions:

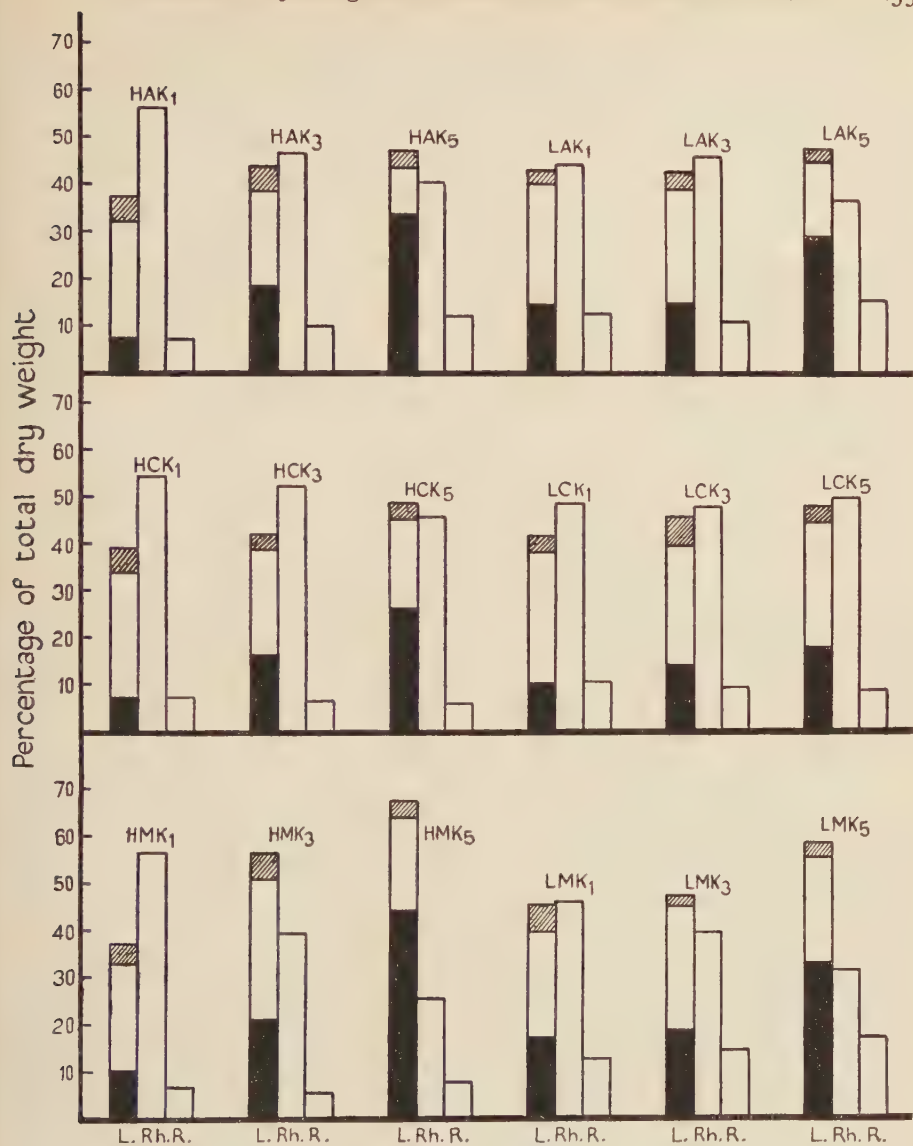


FIG. 2 (c). Total dry-weight distribution: final harvest.

Leaf matter . . . M > C > A
 Rhizome. . . C > A > M
 Root . . . A > M > C

Thus each of the three solutions appears to favour a different fraction. In the ammonium type solution the leaf fraction is especially high, being combined with the lowest rhizome percentage. In plants from the A solution the root fraction is maximal and the leaf fraction minimal, while the high calcium solution

produces the largest percentage rhizome and least roots. From inspection of the growing plants it appeared that those given the M type solution were generally the largest in most of the P and K level combinations. This appearance is now seen to be due to a larger proportion of the total dry weight of these plants having been invested in leaf matter, the only fraction visible above the sand.

The effect of increased phosphorus supply is to decrease the root fraction, mainly to the advantage of the rhizome matter. The $P \times K$ interaction is significant for the leaf and practically so for the rhizome fraction. The higher-order interaction involving also solution type is significant for root and rhizome fractions, and almost so for the leaf fraction (variance ratio = 2.24, 5 per cent. level = 2.37). However, owing to the difficulties of interpretation no detailed discussion of these effects will be given. They can be roughly summarized as follows.

Under conditions of progressive potassium shortage leaf and root fractions generally tend to increase at the expense of the rhizome fraction, but this effect is modified by the simultaneous deficiency of phosphorus, which increases the root fraction; phosphorus deficiency also largely eliminates the differences between the K_1 and K_3 levels, and reduces that between these and the K_5 level. When solution type enters into this interaction the general effect of the C solution of suppressing K-deficiency symptoms makes its appearance again in the elimination of differences between the K_1 and K_3 levels even with high phosphorus supply.

Leaf areas per plant. Leaf areas per plant were measured on the occasions of the first four samples but, with the exception of a single plant from each treatment, not at the final harvest. The spread of the results was such that they needed to be analysed in the form of the logarithmic values of the original figures. They are presented here in this form, but ratios of values cited occasionally in the text refer to the actual areas.

Of all main effects age is the only one which is significant when tested against all its significant interactions with other factors.

The following interactions are significant and will be described: $P \times K$, $P \times$ Solution type, $K \times$ Solution type, and $K \times$ Age. The actual values for these interactions—mean log. per plant—are represented graphically in Fig. 3 (a)–(e).

The significant main effect of age (Fig. 3 (a)) indicates a general increase in leaf area over the period of the first four samples regardless of nutritional treatment. The ratio of the area at the close to that at the beginning of the successive intersampling periods (14 days) are 1.82, 1.14, and 1.25.

$P \times K$ interaction (Interaction diagram, Fig. 3 (c)). Increasing the supply of each of these two elements individually above the minimum quantity leads to large increases in leaf area, the magnitude of the effect rising with increasing levels of the other nutrient. The ratios of the areas at the two phosphorus levels (H/L) are for each of the three K levels: K_1 , 7.73; K_3 , 1.95; K_5 , 1.10.

As has also been shown for total dry weights, a comparison of the two treatment series LK₁ ($= \frac{1}{10}$ th the standard dose of P) and HK₃ ($= \frac{1}{6}$ th the standard dose of K) demonstrates the greater importance of adequate phosphorus supply than potassium supply to the growing bracken plant, i.e. HK₃/LK₁ = 1.4. Again the effect may not be quite general, for, although the higher-order

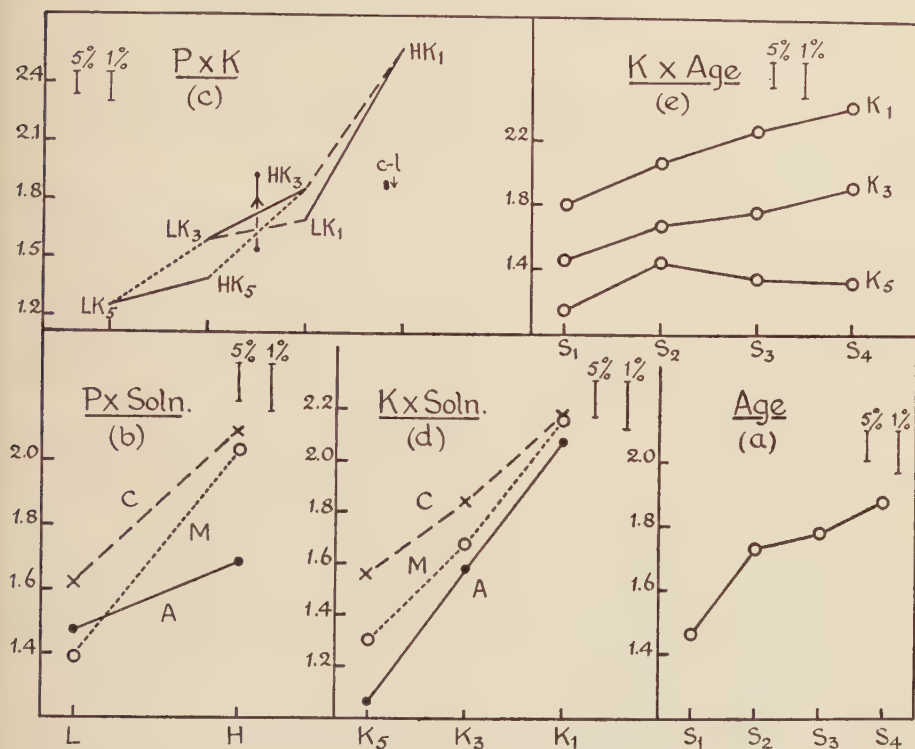


FIG. 3 (a)-(e). Effects of mineral nutrient supply and age on log. total leaf area (cm.²).

interaction of P and K with solution type fails to reach significance level, the ratio HK₃/LK₁ in the A solution is less than unity (0.75), so that here potassium appears to be the more important nutrient.

i. P × Solution type (Fig. 3 (b)). Considering the ratios of high to low P levels in the three solutions it will be seen that the increases in leaf area due to increased phosphorus manuring are greatest in the M and least in the A solution:

	A	C	M
H/L	1.6	2.9	4.3

Thus the leaf area of the HM plants is almost equal to those in the HC series, while the LM treatment carries the least of all. The C solution is superior to the other two at both phosphorus levels but contributes little to this interaction.

ii. $K \times$ Solution type (Fig. 3 (*d*)). In all three solutions leaf areas per plant increase markedly as more potassium is supplied, but the rate of increase depends on the cation composition of the basal medium. The ratios of the different potassium levels for the three solutions are:

	A.	C.	M.
Linear term K_1/K_5	10.4	4.0	7.1
Curvilinear term $K_1 \times K_5/K_3^2$	0.95	1.13	1.29

This interaction is again indicative of a 'buffering' action against some potassium deficiency effects apparently exerted by the high calcium content of the C solution. The high sodium content of the A type, on the other hand, adds to the detrimental effects of potassium shortage.

iii. $K \times$ Age interaction (Fig. 3 (*e*)). The effect of progressing age on the leaf area per plant differs much with varying potassium supply, while the rate of increase of leaf area generally falls with age after the time of the first sample; the effect is least pronounced at high potassium levels. When potassium is in minimum supply, however, the maximum leaf area is attained by the time of sample 2 and falls thereafter, i.e. the rate becomes negative. The K_3 series occupies an intermediate position.

The highest-order interaction involving all three nutritional factors as well as age is itself significant. It would serve no useful purpose, however, to describe this interaction, but it indicates the complexity of the varying effects on one another of the different nutritional treatments with time.

Actually two of the interactions described above, $K \times S$ and $K \times$ Solution, are not significant when compared with this third-order interaction though very nearly so. Hence it seems legitimate to consider them rather than the four-factor interaction, though their generality is not fully established at all levels of the other two factors.

Leaf areas per leaf. Leaf areas per leaf have been calculated from the leaf areas per plant, presented above, and from leaf counts made on the plants sampled. It is interesting to see that the mean area per leaf closely follows the same pattern as total leaf area itself. Previously it has been shown that numbers of leaves are also determined to a large extent by manurial treatment in much the same manner as is the total leaf area. Hence it appears that differences in total leaf area per plant are compounded of two effects, working generally in the same direction: effects on the number of leaves and effects on their individual sizes. Important departures from this correlation are described below.

The data have not been statistically analysed and the original figures are presented (i.e. area in cm^2 per leaf). In quite general terms the size of the average leaf increases with advancing age of plant, and this holds true even when the total leaf area per plant is falling; cf. K_5 level in Table IV, $K \times$ Age, and the corresponding Fig. 3 (*e*) above. Lack of phosphorus leads to greater reduction of individual leaf size than correspondingly severe lack of potassium, i.e. HK₃ v. LK₁. Placing the three solution types in the order of mean leaf

size, we find $C > A = M$. This order differs from similar comparisons noted so far, in which the A type lags much behind the other two. The explanation is probably to be found in the higher leaf death-rate of the sodium solution. Thus, although the total leaf area is smaller than in the other two solutions, it is made up of fewer leaves, produced at a later stage, but which are on the average larger, possibly for just this reason.

TABLE IV
Leaf Area per Leaf (cm.²)

(a)							

material, special standardization with bracken starch to allow for possible differences between the two starches was deemed unnecessary. The maltose produced by hydrolysis was then estimated using Somogyi's (1945) micro-copper reagent. Two sets of controls, consisting of water blanks and the non-hydrolysed starch solution itself, were also titrated.

TABLE V
Percentage Starch in Total Rhizome Dry Weight

(a) Solution A = 41.08 C = 51.86 M = 38.06

(b) Sample S₃ = 34.52 F.H. = 52.81

		H	L
(c)	A	38.94	43.21
	C	51.13	52.58
	M	36.51	39.60

		K ₁	K ₃	K ₅
(d)	A	44.33	43.54	35.37
	C	48.01	52.53	55.03
	M	45.62	41.49	27.06

		H					L		
		K ₁	K ₃	K ₅			K ₁	K ₃	K ₅
A	.	45.54	39.57	31.71	A	.	43.12	47.50	39.02
C	.	45.82	53.21	54.35	C	.	50.19	51.85	55.71
M	.	43.81	42.86	22.86	M	.	47.43	40.11	31.26

The large increase in starch content during the 8 weeks between the two samples is consistent over all treatments, the mean increase being about 53 per cent. of the third sample value. Solution type has a marked effect, the C solution having consistently the highest starch contents at all P and K levels. The potassium effect is also modified by the solution type; thus in the M and A solutions starch content rises with improved potassium supply; in the C solution it increases as the K dosage diminishes. The mean percentage starch is increased in all three solutions when phosphorus is lacking, but the effect is most pronounced in the A and M solutions and when potassium is also reduced to a minimum.

The main facts then which emerge from this consideration of storage carbohydrate contents are: (1) the effects of K shortage in the high calcium solution are directly opposed to those in the A and M solutions; (2) phosphorus-deficient plants have higher starch contents, especially at the K₅ level, the LK₅ plants being superior to the HK₅ ones; and (3) the total starch content at the end of the season reaches the very high value of over 60 per cent. of the dry weight in some treatments.

Leaf and rhizome water contents. Water contents were determined for the green leaves as well as the rhizomes of the bracken plants. The fresh weights of the rhizomes were obtained by washing them free from sand, removing all roots and also the remains of petioles and leaf buds, followed by careful drying of their outer surfaces on a towel.

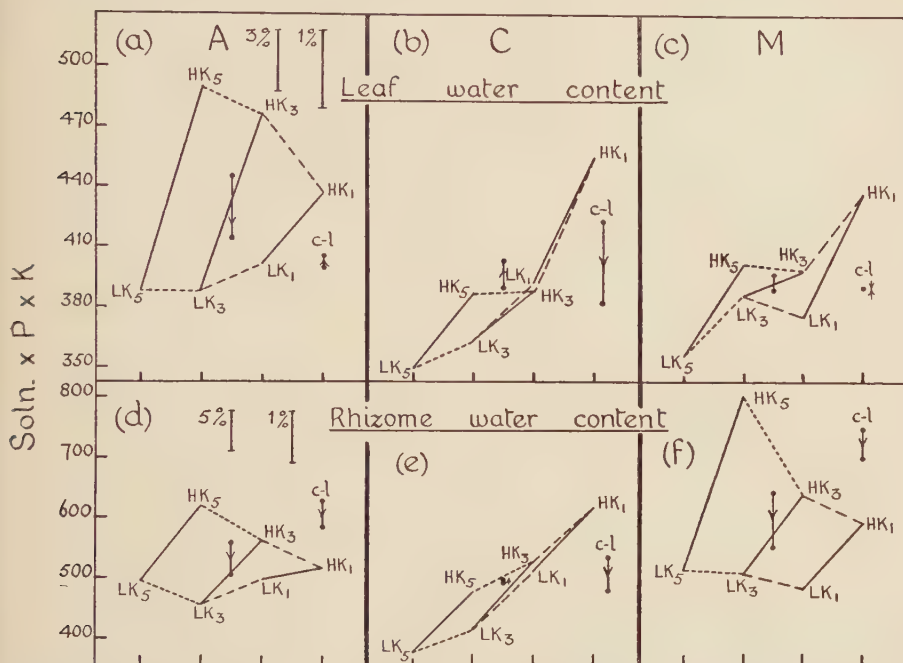


FIG. 4 (a)–(f). Interaction between the effects of phosphorus and potassium supply on leaf and rhizome water contents in three solution types (water content is expressed as percentage dry matter).

Although the age effect on the water contents of the rhizomes differs somewhat from that on leaf water content, both fractions are considered together as all nutritional effects appear to be closely similar (Fig. 4 (a)–(f)).

The most striking nutritional effect is the great reduction in water content due to phosphorus starvation, which amounts to 20 per cent. of that in the H series in the case of the rhizome and 12 per cent. for the green leaf matter. The difference between the leaf water contents at the two phosphorus levels is affected largely by age of plant; it diminishes with time, so that it has vanished by the fourth sample, only to be re-established by the final harvest.

In the rhizomes the mean age effect itself is very large indeed. The fall throughout the season is rapid and by the final harvest time the mean water content of all nutritional treatments, apart from K₅, is less than 50 per cent. of that at the first sample (Fig. 4 (h)).

The Solution type $\times$ P $\times$ K interaction is significant for both leaf and rhizome water contents (Fig. 4 (a)–(f)).

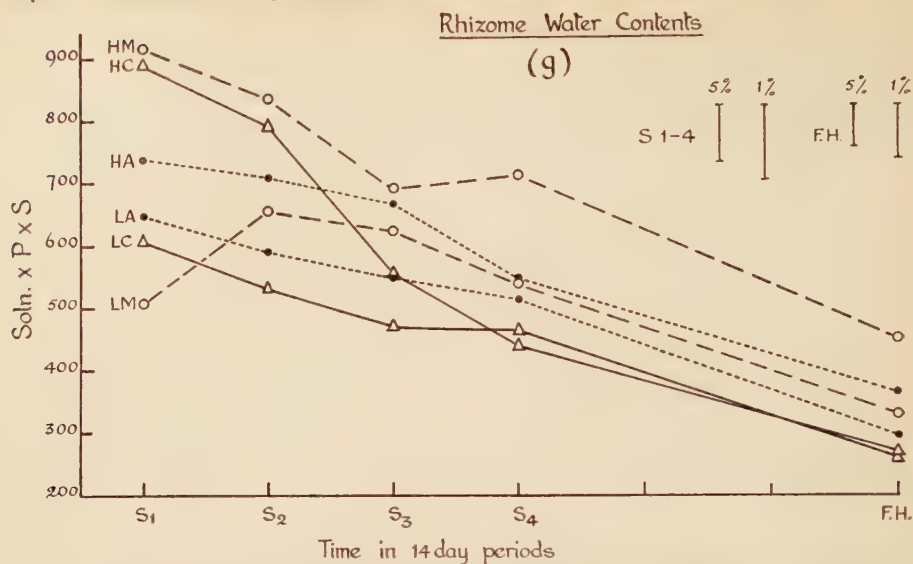


FIG. 4 (g). Effects of phosphorus level and solution type on rhizome water contents at different ages of plant (percentage dry matter).

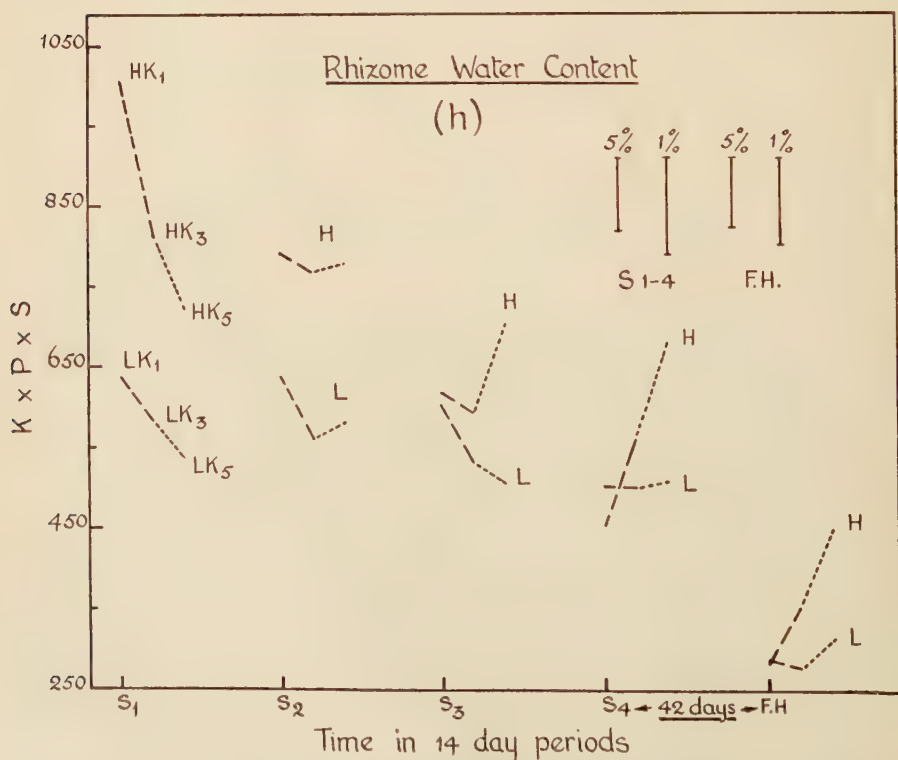


FIG. 4 (h). Effects of phosphorus and potassium supply on rhizome water contents at different ages of plant (percentage dry matter).

In the case of the leaf water contents significance is mainly due to the following opposing tendencies: At the higher phosphorus level succulence falls with decreasing K supply in the C and M solutions, but rises in the HA series. When phosphorus is reduced all values are lowered considerably, and diminished K supply causes still further decreases of succulence in the C and M types as before, but the LA series are now affected little by potassium level.

This interaction is substantially repeated for the rhizome water contents, with one important difference. The M solution effect is quite reversed, i.e. it follows a similar course to that in the A solution, showing significant increases with diminution of potassium dosage at the high phosphorus level.

The rate of diminution of succulence of the rhizome with progressing age is much affected by the $P \times \text{Soln.}$ interaction. Thus water content diminishes more rapidly in the C solution than in the other two at both high and low P levels. In the HM series the fall is apparently arrested altogether between samples 3 and 4, and in the LM series there is even a significant initial rise (between samples 1 and 2) followed by a slow fall (Fig. 4 (g)).

The $K \times P \times S$ interaction on rhizome water contents is also highly revealing. The dependence on K supply of the general age and phosphorus deficiency effects (reduced succulence) is seen in Fig. 4 (h). Both HK₁ and LK₁ treatments are initially more succulent than the corresponding K₃ and even the K₅ series, but differential rates of fall in water content cause a complete reversal of this situation by the fourth sample at the H level and by the final harvest at the L level. The change in the relative position of the K₅ series actually starts as early as the second sample.

In addition to the interactions described the $\text{Soln.} \times K \times S$ interaction on leaf water contents and the highest-order interaction on rhizome water contents, involving age as well as all nutritional factors, have been shown to be significant at the 1 per cent. level. However, since the effects are not easily visualized a detailed description will not be attempted. But, since the two interactions on rhizome water contents described above are not significantly larger than this higher-order interaction, the proviso must be added that each is modified significantly by the fourth factor, i.e. the $\text{Soln.} \times P \times K$ interaction by age (S) and the $\text{Soln.} \times P \times S$ interaction by K level. Thus the demonstrated relations between the effects of the investigated factors on rhizome water contents are highly complex and completely valid generalizations as to these effects are largely confined to the main effects of P supply and age.

Net assimilation rates. Net assimilation rates (N.A.R.) have been calculated for the three intersample periods between S₁ and S₄; they are shown in Table VII. Since they are calculated from figures involving increments in both leaf area and dry weights, they are of necessity subject to large errors. With a rather variable plant like bracken, error is increased even further and only large treatment differences are likely to reach significance. For these reasons the results have been analysed in two ways: (1) using the values for the first and third intersample periods only, omitting the second since this is dependent on the other two; (2) using the mean N.A.R.s calculated for the

whole 6 weeks from S₁ to S₄, i.e. rates calculated from the data collected at samples 1 and 4 only. In the first analysis the third-order interaction was used as error, in the second the pooled variances of all first- and second-order interactions.

The figures given are mg. dry weight per cm.² per week. To convert this value into mg. CO₂ per dm.² per hour they need to be multiplied by 1.42. (In the calculation of this conversion factor all dry-weight increases were assumed to be in the form of hexose sugars, and 14 hours 45 minutes, i.e. the mean day length between the first and fourth samples was chosen as the period of effective daylight.)

The following effects have been found significant: Net assimilation rate is severely reduced when phosphorus supply is inadequate. The effect of potassium shortage is to depress the rate very severely in the K₅ series, but there is no appreciable indication of any effect at the K₃ level. Unfortunately net assimilation rates are particularly variable under conditions of potassium deficiency and when the second intersample period is omitted and the first and third are considered alone, the whole effect of K supply disappears. Nevertheless there can be little doubt of its reality.

TABLE VI
Net Assimilation Rates (mg./[week/cm.²])

Treat- ment.	Inter- sample period.	N.A.R.	Treat- ment.	Inter- sample period.	N.A.R.	Treat- ment.	Inter- sample period.	N.A.R.
HAK ₁	I	5.31	HCK ₁	I	6.64	HMK ₁	I	5.49
	II	3.31		II	5.70		II	5.42
	III	3.20		III	6.42		III	4.12
HAK ₃	I	11.31	HCK ₃	I	5.15	HMK ₃	I	3.52
	II	-0.29		II	3.23		II	2.76
	III	7.61		III	10.15		III	3.08
HAK ₅	I	15.93	HCK ₅	I	3.70	HMK ₅	I	5.55
	II	-9.30		II	1.34		II	1.49
	III	1.16		III	4.66		III	0.18
LAK ₁	I	2.16	LCK ₁	I	3.75	LMK ₁	I	4.07
	II	4.04		II	3.22		II	2.71
	III	4.56		III	3.20		III	3.81
	I	5.82	LCK ₃	I	1.20	LMK ₃	I	4.30
	II	3.29		II	3.13		II	4.39
	III	-0.32		III	1.14		III	6.67
LAK ₅	I	3.70	LCK ₅	I	5.47	LMK ₅	I	4.26
	II	2.06		II	1.81		II	1.59
	III	-2.71		III	6.75		III	-1.44

Net assimilation rate falls significantly from the first to the third intersample period, the drop exceeding 35 per cent. The interaction of solution type and sampling time is the only other effect to be noted. The rate of the A and M solutions falls very steeply from the first to the third intersample,

particularly in the A solution, while that in the C solution actually appears to increase, but not significantly so. Inspection of Table VI also shows that this difference between the effects of the three solutions is consistent over most P and K levels.

pH of solution in sand culture. The effect of differing acidity of the medium on the growth of bracken in very similar sand-culture conditions to the main experiment was tested in a small preliminary experiment, the pH values employed being 4.0, 5.5, 7.0.

Once a week each pot was irrigated from a reservoir of nutrient solution until the outflowing solution was of the desired pH, 2-3 irrigations being required. The weekly shift in pH exceeded 1.5 units only on a few occasions. The plants were harvested after 2 months. Unfortunately the plant material in this experiment was rather variable and no significant differences in dry weight were found. The mean total dry weights per plant were: pH 4.0, 11.05 g.; pH 5.5, 11.60 g.; pH 7.0, 8.55 g., the error being ± 3.13 .

Confirmatory evidence is afforded by the pH of the culture solutions in the main experiment. As has been pointed out in Part I of this paper these were adjusted to pH 5.5 in the A and M solutions and found to be at 4.0-4.5 in the C solution. These values were increased somewhat by the residual alkali of the tap-water used in washing the sand. pH determinations on samples of culture solution withdrawn from the pots on the occasion of the first and second growth samples and the final harvest are shown in Table VII.

TABLE VII

pH of Solution: Means of Significant Effects

				S ₁ .	S ₂ .	F.H.
H	K ₁	A	.	7.77	7.58(7)	7.95(8)
		C	.	7.03	6.74	7.43
		M	.	5.95	5.91	5.39
	K ₃	A	.	7.63	7.55	8.12
		C	.	6.96	6.90	7.18
		M	.	6.88	5.87	3.45
	K ₅	A	.	7.78	7.51	7.83
		C	.	7.16	7.07	7.24
		M	.	6.15	5.67	3.87
	L	K ₁	A	.	7.89	7.84
			C	.	7.72	7.49
			M	.	7.01	5.95
		K ₃	A	.	7.92	7.81
			C	.	7.63	7.51
			M	.	7.07	6.21
		K ₅	A	.	7.91	7.71
			C	.	7.84	7.48
			M	.	7.13	5.92

Significant difference between any pair of values: 0.99 for S₁ and S₂ and 0.70 for F.H.

Little need be said in explanation of these figures. The differences in pH value between the several treatments are, of course, confounded with the nutritional composition of the solution, but the good growth obtained in the C solution (extreme value 7.9) and the M solution (extreme value 3.1) indicate the marked tolerance of bracken to a wide range of H-ion concentration in its growth medium.

The main nutritional effect, lowering of pH in solutions containing ammonium nitrate (owing to the faster uptake of the ammonium ion), and increases of pH in solutions containing any nitrate, is, of course, well known from similar experiments with other plants.

COMPARISON OF N- AND P-DEFICIENCY EFFECTS (*Subsidiary Experiment I*)

In order to determine the relative severities of nitrogen and phosphorus deficiencies a small-scale experiment was set up using distilled water only. The treatments comprised three levels of phosphorus and three of nitrogen, not combined factorially: N1P1 the standard quantities of both elements; N1P3 = $\frac{1}{9}$ th and N1P5 = $\frac{1}{81}$ st of the standard amount of phosphorus; N3P1 = $\frac{1}{9}$ th and N5P1 = $\frac{1}{81}$ st of the standard amount of nitrogen. Each treatment was replicated six times.

Methods of raising the plants and dates of planting out, &c., were identical with those in the main experiment. The sole difference lay in the fact that the sand was washed several times with distilled water. The basal solution was of the same composition as that applied during early stages of growth in the main experiment, a mixture of equal parts of the A and C solutions. The pH was adjusted to approximately 5.5 by addition of H_2SO_4 . Sodium and calcium levels of the deficient treatments were made equal to those of the high nutritional treatments by addition of sodium sulphate and calcium chloride, so that all unwanted variability in nutrient composition was thrown upon the chloride and sulphate ions. All plants were harvested after 4 months.

Results. The first symptoms of both nitrogen and phosphorus deficiencies began to appear 3 weeks after applying the differential treatments.

The nitrogen-deficient plants, especially those at the lowest level (N5), showed a marked yellowing of the leaves and a lag in general growth. After a further 3 weeks all the larger leaves of the N5 plants had died and the new leaves being produced were minute in size. Deterioration continued rapidly until, only 2 months after planting out, all the N5 plants were dead. The nitrogen-deficient plants at the N3 level, however, continued to grow comparatively well; their leaves were rather yellow, hard, brittle, and few in number, but compared with the controls the relative proportion of dead leaves was not increased.

Phosphorus-deficiency symptoms were similar to those in the main experiment; however, plants grown at the lowest P level (P5) showed them with increased severity. Thus the proportion of dead leaves was much increased and at harvest the plants bore only a few living leaves of very small size. The numerical data collected, both primary and derivative, were the same as in

the main experiment summarized in Table VIII. The following points may be noted:

1. The early death of all plants at the lowest N level.
2. Reduced leaf production in all deficient treatment, especially in the P₅ plants.
3. Increased longevity of leaves at the N₃ level—the greatest found in any treatment throughout these bracken experiments. The slight increase of longevity of leaves seen at the P₃ level (confirming a similar effect in the L treatment of the main experiment) is not maintained when phosphorus is further reduced to $\frac{1}{81}$ st of the standard quantity.
4. Severely reduced dry weights in all deficient treatments, N₃ and P₃ levels having about the same effect.
5. Fractionation of the dry weight indicates a progressive increase in the proportion of leaf matter with decreasing phosphorus and a decrease in leaf matter when N was at a minimum. Phosphorus deficiency causes a considerable reduction of percentage rhizome matter, but the N₃ plants do not differ from the controls in this respect. The root fraction is increased in all deficient treatments.
6. Starch content of the rhizome is reduced at the lowest level, P₅, but to a value that is still quite high relative to that found at the $\frac{1}{81}$ st K level in the main experiment, i.e. 36.2 per cent.

TABLE VIII

	Standard N ₁ P ₁ .	N ₁ P ₃ .	N ₁ P ₅ .	N ₃ P ₁ .	N ₅ P ₁ .
Total number of leaves produced . . .	41	33	22	29	—*
Living leaves when sampled . . .	19	11	3	14	—*
Mean longevity of leaves (weeks) . . .	6.7	7.3	6.4	8.3	—*
Total dry weights (g.) . . .	15.35	4.92	0.806	4.61	0.088
Dry-weight distribution (%): Leaves . . .	37.7	40.6	44.5	31.7	—*
Rhizome . . .	54.0	44.0	31.9	54.0	—*
Roots . . .	8.3	15.4	23.6	14.3	—*
Starch content (% dry weight) . . .	54.4	56.5	46.6	56.9	—*

* Not recorded owing to premature death of plants.

It appears then that equal moderate reductions in the levels of phosphorus and nitrogen lead to similar decreases in the total size of the plants, but the distribution of the dry matter is altered differentially. At extreme deficiency levels nitrogen-starved plants are killed, but those lacking phosphorus may survive, at any rate through the first growing season.

SHADING *v.* POTASSIUM DEFICIENCY (*Subsidiary Experiment II*)

Some observations and a preliminary experiment carried out during the previous season had indicated a beneficial effect of shading on bracken plants suffering from acute potassium shortage. In order further to test this effect

an experiment was set up in 1947 in which bracken plants were grown in an unheated greenhouse at high and low levels of potassium and in two light intensities. The control plants were grown in full daylight, the shaded set under butter muslin held by wire supports; the basal nutrient solution was intermediate between the A and C types and the two potassium levels were 1.867 and 0.086 g. per pot respectively. Soon after the beginning of the experiment one of the four replicate plants in each of the four treatments died accidentally and was replaced by stock plants of the same age.

Results. The observational results agreed fully with those noted in the previous seasons. The leaf death-rate of the deficient plants was much retarded; in the shaded set their appearance was darker green and generally more healthy than that of the full-light controls. Their size was also markedly larger. Of the high potassium controls, on the other hand, those in the full light showed much more vigorous growth and finally reached a much larger size than the shaded ones. By the end of the experiment two of the potassium-starved plants exposed to the full light had died after exhibiting K-deficiency symptoms of the greatest severity. The deficient shaded plants, on the contrary, though somewhat etiolated, presented a much more normal appearance and all survived.

The mean logarithms of total dry weight (expressed in decigrams) of the treatments are given below:

TABLE IX
Logarithms of Total Dry Weight

	Fully manured.		K-deficient.	
Light	.	.	2.452	1.002
Shaded	.	.	2.060	1.884

Significant difference = 0.344

The statistical analysis of these figures indicates that the interaction is well above significance level.

The water contents of leaves and rhizomes, as percentage of the dry weight, were as follows:

		Leaves.		Rhizome.	
		F.M.	K-deficient.	F.M.	K-deficient.
Light	.	422.0	427.6	372.1	559.6
Shade	.	526.6	495.3	447.3	361.5

It is seen that shading increases the water contents of all leaves but of rhizomes only at the high potassium level; in the K-deficient treatment rhizome water content is much below that of the controls in full light. These differences in rhizome behaviour must largely reflect differences in starch content.

The dry-weight distribution is also of interest. The figures below are percentages of the total.

TABLE XI

			Leaf matter.	Rhizome.	Roots.
F.M.	{ Light . . .		52.5	30.0	17.5
	{ Shade . . .		67.8	25.7	6.5
K-deficient	{ Light . . .		64.5	17.6	17.9
	{ Shade . . .		68.0	26.7	5.3

The main effect of shading is to decrease to very low values the percentage root fraction; conversely, percentage leaf matter is always increased, but the effect of shading in percentage rhizome varies with potassium level: at high level a reduction may be found, but at the low level a considerable increase occurs. Under shade conditions potassium level has no appreciable effect on the dry-weight distribution, but at high light intensity potassium deficiency leads to a lowered percentage rhizome and increased percentage leaf matter.

Conclusions. It is evident from these data that bracken plants grown under conditions of potassium shortage with sodium present (B solution) benefit materially from being shaded. External symptoms of deficiency are much reduced, in particular leaves exhibit few signs of chlorosis and though slightly etiolated are almost normal in appearance. Concomitantly with the reduction of deficiency symptoms, more growth is made by shaded plants and much higher dry weights are reached.

In spite of the small size of the experiment, then, its rather unexpected results are noteworthy.

DISCUSSION

Although the bracken plant differs considerably in its growth habits from barley and flax it will be seen from the comparisons given in this discussion, especially in the final section, that it responds in a very similar manner to identical nutritional treatments. This similarity is important since it confirms the general validity of the results obtained with barley and flax by extending them also to a cryptogam.

The following discussion is subdivided according to the different aspects of the plant's metabolism: (1) Carbohydrate metabolism, including assimilation, dry-matter accumulation, distribution, and storage; (2) Nitrogen metabolism, including meristematic activity, synthesis of new protein, and the form of nitrogen presented; (3) Toxicity effects and protein maintenance, water contents, and pH effects; (4) Comparison between the effects of identical nutrient conditions on barley, flax, and bracken.

1. *Carbohydrate metabolism.* Apart from small errors due to mineral element content, the total dry weights represent the net result of carbohydrate metabolism. An attempt is made below to analyse further the differences in total dry weight between the various nutrient treatments. Dry-matter changes

over a given period can be accounted for by net assimilation rate, a measure of efficiency representing the balance of photosynthetic gains and respiratory losses per unit leaf area and time, and by the mean leaf area. The mean leaf area itself is the product of leaf number and the average area per leaf; while the number of active leaves is determined by their rates of formation and death (meristematic activity and longevity). In order to assess roughly the relative importance of these factors, the N.A.R., the mean leaf number, and the average area per leaf for the eighteen treatments have been tabulated (Table XII) side by side with the total dry weights. The values within any one column are expressed as percentages of the mean over all treatments, the mean value itself being given at the head. The dry-weight figures refer to the fourth sample, while N.A.R.s, &c., are the means over the preceding period from samples 1-4.

TABLE XII
Percentage of Mean of Column

Treatment.	Total dry weight (g.)	N.A.R.	Leaf No.	Area per leaf (cm. ²).
Mean value	4.10	3.95	11.1	7.43
HAK ₁	234 %	119 %	144 %	238 %
HCK ₁	460	171	170	310
HMK ₁	493	142	221	296
HAK ₃	56	191	58	85
HCK ₃	154	143	110	179
HMK ₃	58	87	123	74
HAK ₅	11	46	36	40
HCK ₅	35	90	67	78
HMK ₅	22	64	72	55
LAK ₁	45	89	107	65
LCK ₁	46	85	121	62
LMK ₁	36	93	107	48
LAK ₃	29	88	90	46
LCK ₃	34	77	101	55
LMK ₃	33	120	93	46
LAK ₅	12	28	50	32
LCK ₅	34	108	69	67
LMK ₅	11	56	62	23

In using the table comparison can be made immediately for each treatment between the relative magnitudes attained by the three characteristics in the row. Naturally this method is subject to large errors and it will be seen that, in general, the factors tend to vary together—a fact which serves to emphasize their close interdependence, but renders the assessment of their relative importance more difficult. Nevertheless, important deviations accounting for the greater part of the observed effect on total dry weight may be pointed out.

In the three control treatments HAK₁, HCK₁, and HMK₁, the large size of the individual leaves is mainly responsible for the high dry weights,

deviations in leaf number and N.A.R. being considerably smaller, although in the ammonium solution leaf number is also becoming important. In the HAK₃ treatment the very high mean net assimilation rate as calculated cannot be accepted as real since the basic measurements in this treatment were particularly subject to error owing to the rapid death of leaves, &c. (cf. also Table VI). On the other hand, the number of living leaves present at any time is severely reduced. Of the other HK₃ treatments the M solution again has large numbers of leaves, but of reduced size; in the C solution leaves are somewhat

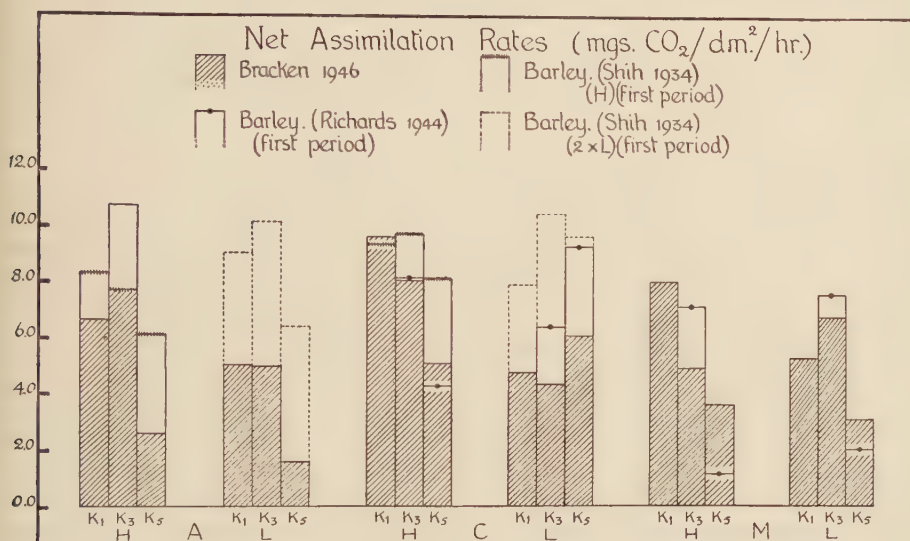


FIG. 5. Comparison of the net assimilation rates of barley and bracken receiving similar nutritional treatments.

fewer but much larger, also the N.A.R. is high. In the three HK₅ treatments all characteristics are substantially reduced, but not markedly differentially, though there is again evidence that, as at the K₃ level, in the M solution more leaves are produced than in the C, and that they are smaller.

Throughout the phosphorus-deficient series leaf size is very consistently the factor showing greatest reduction, the only slight exception being the LAK₅ treatment in which N.A.R. is also very low indeed. Leaf number has been relatively least affected, although the absolute effect of P shortage on 'meristematic activity' itself (as assessed from leaf and bud numbers in comparison with the HK₁ treatments) is very large also. The comparatively high N.A.R. shown for the LMK₃ and LCK₅ treatments are perhaps surprising, but there is nothing to suggest unusually large errors here, as in HAK₃, and they must be regarded as real; under the same conditions barley also shows relatively high N.A.R.s (Fig. 5). The nutrient effects on these factors controlling total dry-weight production may then be roughly summarized as follows: though the effects of K shortage are variable in bracken at the K₃ level, N.A.R.s are

severely curtailed in the K₅ series. Restriction of P supply to $\frac{1}{10}$ th caused a reduction of about 25–50 per cent. The form of nitrogen supplied may also be of some importance in this respect. Vickery (1940) found some reduction of photosynthetic activity in tobacco supplied with NH₄ as compared with that of plants given nitrate; slight differences between the HMK₁ and HCK₁ bracken series or the means of the M and C series tend to confirm this observation, but neither effect reaches significance level.

Real assimilation rates of deficient barley leaves were measured directly by Gregory and Richards (1929) and found to be clearly subnormal. When potassium is lacking this effect is further accompanied by an increase in respiration rate per unit dry weight. The respiration of phosphorus-deficient plants calculated on this basis is, however, decreased, and on a dry-matter basis effects of a reduced real assimilation rate are here somewhat offset by reduced respiratory losses.

The active leaf area as determined by leaf number and mean area per leaf is largely controlled by the use made of the fresh assimilate. The potential increase in leaf area may be limited in two ways: (1) by storage of assimilate or its use in ways that do not increase the assimilatory capacity of the plant, and (2) by premature death of leaves, involving the necessity of their continuous replacement. There is some evidence from subsidiary experiment I that such storage of unutilized carbohydrate takes place in the N-deficient plants where the leaf fraction of the total dry weight is the lowest of all treatments in all experiments. Both rhizome and root fractions are here high. Richards (1944) suggested that only such carbohydrate as is not diverted to the developing leaves and branches is available to the roots, whose fraction of the whole is thus inversely proportional to the meristematic activity and growth rate of the tops. The second factor (a disproportionate amount of assimilate being required to maintain the leaf area) is important in the K-deficient series where increased death rate of leaves causes a rapid loss of carbohydrate—already at a minimum—in the dead organs. A correlation diagram between the percentage dead leaf matter and log. total dry weight reveals a close inverse relationship between these two characteristics. (Fig. 6.)

The starch contents of the rhizome in the different treatments also give an indication of the fate of the fresh assimilate of the bracken plant, reflecting at the same time differences in N.A.R.s. In treatments wherein nutrient deficiency does not check meristematic activity primarily, rhizome starch content generally falls with diminished N.A.R.s, i.e. the K-deficiency treatments in the A and M solutions. But reduced N.A.R.s do not necessarily lead to low internal carbohydrate levels; and in the phosphorus-deficient series ($\frac{1}{10}$ th) where meristematic activity is reduced, quite high starch contents are found, though the amount of rhizome matter relative to the whole plant is reduced. However, when P supply is curtailed to $\frac{1}{81}$ st of the standard dose, rhizome starch content is reduced again by about 14 per cent. In the C solution also potassium deficiency depresses N.A.R. (though to a lesser degree than in the other two solution types), but starch contents are as high or higher than

in the controls. The explanation for this difference between the three solutions is likely to be due to a combination of effects on meristematic activity and toxicity effects; all three solutions show reduced meristematic activity but only the A and M solutions produce toxicity symptoms.

2. *Nitrogen metabolism.* The results presented cover only some aspects of

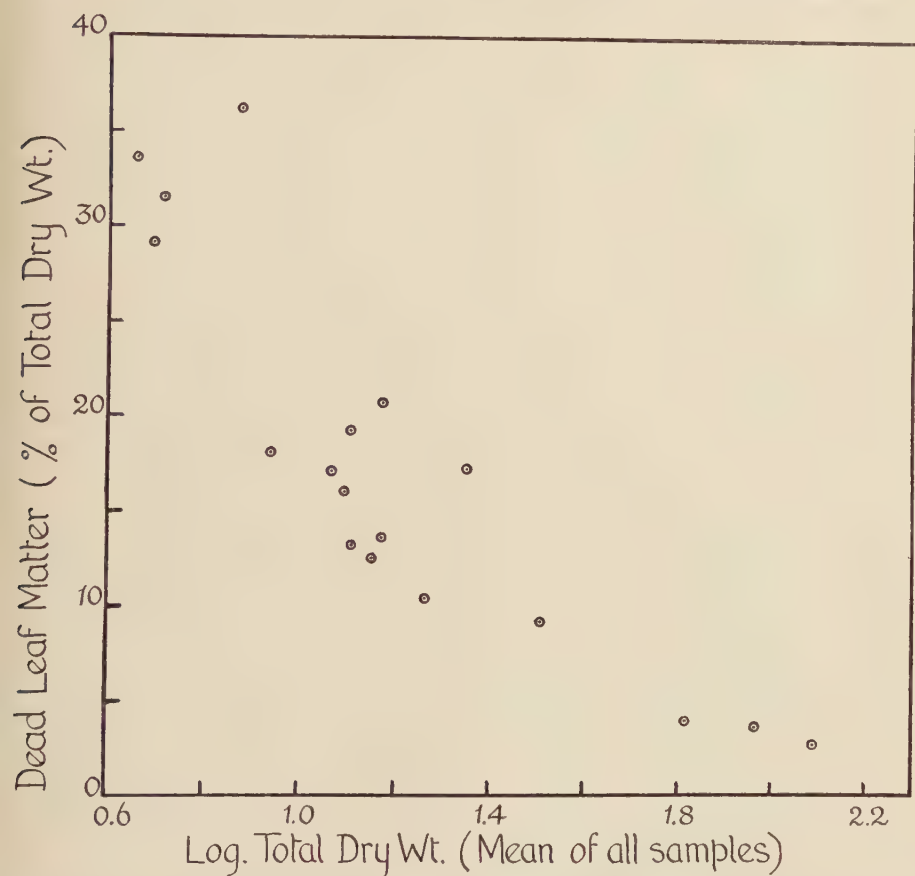


FIG. 6. Correlation between percentage dead leaf matter and log. total dry weight.

the nitrogen metabolism and this indirectly. The data on rates of leaf production, leaf and rhizome bud numbers serve as a measure of the meristematic activity of the plant. Of course, counting the numbers of meristematic centres is not an ideal method of estimating masses of meristematic tissue, e.g. the large nutritional effects on leaf size are probably due in the main to differences in cell numbers and only partly on cell dimensions. On the other hand, the correlation between leaf numbers per plant and their individual size is generally very strong, so that bud numbers, &c., present a simple first basis for comparing the degree of meristematic activity. In the barley experiments at this Institute tiller counts have been used for the same purpose.

As has been frequently demonstrated for many plants, nitrogen and phosphorus deficiencies are here seen to limit meristematic activity, and this may be taken as implying reduced protein synthesis partly by way of lack of the constituent elements, but presumably also indirectly by restricting other processes like respiration.

Superimposed on these effects, Richards (1942) noted an increase in apical dominance in P-deficient flax cultures. In bracken a similar effect may well have contributed to reduced numbers of meristematic centres, but it can only be distinguished in plants with well-defined cycles of shoot production, &c.

Effects of potassium shortage on meristematic activity are not so clear cut. Consideration of the present experiment alone would indicate that in the absence of toxicity effects of other ions (cf. below) potassium deficiency, like those of N and P, also limits the number of leaf buds and rhizome branches together with individual leaf size (HCK5 and LCK5). A similar effect on tiller number obtains in barley, when grown in the C solution deficient in K, but not when grown in an equally deficient solution of the A type. Here tiller numbers are reduced only during the early stages, this being followed by increased tillering, usually in recurring cycles. This activity is accompanied by large toxicity effects which would suggest that it is a consequence of the premature death of the older tillers, i.e. complete destruction of apical dominance. This would also serve to explain its cyclic occurrence. In flax also, potassium deficiency causes very large increases in axillary shoot development in all three solution types, and again apical dominance seems largely lost. It is clear from this that when apical dominance is eliminated by lack of K, meristematic activity can exceed that of fully manured plants. Thus, though lack of K may limit meristematic activity directly in all three solutions for bracken, and in the C solution in the case of barley—the effect is small compared with the inhibition due to ordinary apical dominance and is likely to be indirect. Conversely, if in barley grown at high K levels in the A solution apical inhibition could be neutralized still higher tiller numbers might result, or vice versa a strengthening of apical dominance at low K levels by other means (such as reduced illumination) might considerably prolong the life of a smaller number of tillers (cf. below).

The form of N supplied is also of importance in determining the rate of meristematic activity. In the fully manured ammonium series leaf numbers, rhizome branches, and leaf buds are increased, though the statistical analysis carried out with bud numbers does not show this effect to be significant. In bracken the effect is less marked than in flax, where a large increase in the number of side shoots is highly significant. A small water-culture experiment with young bracken plants also demonstrated that the NH_4 ion is taken up much more rapidly than NO_3 ; and an ammonium source may lead to much higher N levels in the plant (cf. also Vickery, 1940, and Tiedjens and Robbins, 1931). Gregory (1937) and Crowther (1934) have suggested that in the normal cereal plant a stage is reached when internal starvation supervenes, and that at this time further tillering and branch production is suppressed. The

increased internal N content and higher rate of uptake from an ammonium solution may delay the time at which this occurs. In flax fertility is depressed under these conditions. Furthermore, it is possible to induce all these phenomena by heavy applications of nitrate late in the growth cycle.

Before leaving the matter of meristematic activity consideration should be given to the rather unusual observation that all plants suffering from extreme nitrogen deficiency (N₅) in the subsidiary experiment I (p. 246) died prematurely. Nitrogen deficiency usually has no lethal effect and deficient plants tend to live for a long time, though much dwarfed; even in this experiment plants suffering from less severe starvation at the N₃ level exhibited considerable longevity. As was described in Part I of this paper, the plants were raised at a high nutritional level and transferred to deficiency conditions at planting out, and the drastic change itself would appear to be a possible cause for the death of these plants. However, equally great reductions of K and P had no lethal effects. A more plausible suggestion is perhaps that this transfer occurred at the time when the plants were changing from an upright mode of life to the rhizomatous habit, and the demands of the young rhizome may have been so large as to leave insufficient N for the production and expansion of new leaves (only minute initials were found on the dead plants). It appears from other observations that the rhizome is generally a more successful competitor than the leaves for materials in short supply, e.g. (1) in all cases where plants died owing to deficiency of potassium, the rhizome—and every part of it—remained alive until the last leaf had died, and (2) the internal carbohydrate level of the AK₅ treatments was presumably much reduced (extrapolation from barley analyses) and yet the rhizome was able to accumulate starch up to 43 per cent. of its dry weight.

3. *Toxicity effects and maintenance of protoplasm.* Premature death of leaves usually preceded by the formation of local necrotic areas is a well-known and perhaps the most obvious symptom of K deficiency. Richards and Shih (1940) have shown for barley that the severity and details of these symptoms are not constant for any given K level but vary according to the presence of other ions. Similar differences were noted in the case of bracken in the first part of this paper. These symptoms are most pronounced in the A and M solutions and when P is in excess, i.e. those potassium-deficient treatments which are most detrimental to barley. This apparent failure of protoplasmic stability and the high respiration rates associated with it were fully discussed by Richards and Templeman (1936). They came to the conclusion that since protein synthesis in new organs was not markedly affected, it must be the maintenance of the existing protoplasm which was interrupted by the lack of K. They also argued that this must be due to an enhanced rate of protein breakdown rather than inhibition of replacement synthesis. Shcherbakov (1946) and other authors also have actually found increased protease activity in plants suffering from K deficiency. The actual cause for this accelerated breakdown has been ascribed by Richards to the accumulation of 'toxic' agents. There is good evidence that in barley at least very high concentrations

of P may be accumulated under such conditions. In the A solution excessively high Na concentrations have also been found in barley, and in the ammonium type solution it is the NH_4 ion which tends to reach toxic levels. It is not certain whether the presence of excessive amounts of certain ions is directly responsible for the toxicity effects or whether their action is more indirect. Recently Steinberg *et al.* (1950) have suggested on the evidence of feeding experiments that accumulation of certain amino-acids under deficiency conditions may be the cause of the symptoms observed.

Of these three possible types of toxicity rather less evidence for harmful excess P uptake has been found in bracken than in barley. Only in the A solution are the total dry weights higher when phosphorus supply is reduced at the lowest K level, and even here the effect is slight. The markedly increased longevity of leaves in P-deficient plants is also found at the AK1 level, though even here P uptake may have been super-optimal.

The problem of ammonium toxicity in K-starved barley has been discussed by Richards (1941*b*), and he has shown that some at least of the detrimental effects noted can again be accounted for in terms of excess phosphorus accumulated by the barley plant. In bracken, on the other hand, it would appear that ammonium toxicity is certainly dominant. Here under conditions of reduced meristematic activity imposed by lack of P (LMK1) dry weights are much more reduced than in the other two solutions, and leaf longevity is decreased slightly. It is suggested that ammonium toxicity is the factor responsible. Rippel *et al.* (1933) and Viets *et al.* (1946) have noted under K-deficiency conditions considerably increased internal concentrations of free ammonia (up to 10 per cent. of the total N). The detrimental effect of reduced phosphorus supply on bracken grown in the ammonium solution is discernible even at the lowest level of potassium, and it seems that any hypothetical improvement due to elimination of P toxicity must have been masked entirely by increased ammonium toxicity.

The growth of bracken in the high sodium solution was inferior to that in the other two solutions whether comparison is made between the average yields over all treatments or only among the HK1 series. This effect becomes progressively more severe with age and must probably be attributed to toxically high internal Na concentrations. It is known that K-deficient barley can accumulate up to 8 per cent. NaO of its dry weight and other plants are also known to do so, e.g. soy bean (Sayre *et al.*, 1947). Certain other possible explanations of the effect can be excluded for the following reasons: (1) Na limiting K uptake—trebling the dose of K applied resulted in no benefit. (2) Lack of Ca—equal to supply in M solution: also Ca deficiency failed to appear in a sand-culture experiment designed expressly to this end. (3) High pH of external solution—equally high pH in C solution. Sodium toxicity therefore would appear to be the most plausible explanation.

As was seen in the subsidiary experiment described above, light intensity is another factor interacting with potassium effects. This interaction has not been subjected to a great deal of systematic investigation. Previous work has

centred mainly on the question whether a high K supply can compensate for lack of light, i.e. it has been suggested that K manuring is more effective in dull seasons (Rothamsted, 1927-8; Bewley, 1929). Other investigations have yielded conflicting results, but were negative on the whole. Frank (1936) failed to detect any favourable effect of increasing K supply in shaded plants. In the heavier shade he frequently found even a reduced K concentration in the leaves if this was expressed on an area basis.

More recently t'Hart (1949) has stressed the importance of temperature on the growth response to potassium manuring. He found that K manuring of grassland increased yields only at low temperatures (10°C.), but not at higher temperatures (19°C.).

The results of the bracken experiment indicate that shading will reduce some of the detrimental effects of potassium deficiency. This improvement is dependent upon the K level supplied, becoming less obvious as the supply is raised. A similar result was obtained with barley in a small outdoor experiment carried out by the writer. A further large-scale investigation of this interaction under greenhouse conditions by Parkinson (1950) has revealed also beneficial effects on chlorophyll content, fertility, &c., of barley plants, but under these conditions total dry weights of the K-starved plants were not increased by shading as was the case in the bracken and the preliminary barley experiments.

Two possible causes suggest themselves for the beneficial effects of shade on such plants which (extrapolating again from earlier barley analyses) are already severely carbohydrate starved. The first is that the improved appearance and green colour of the leaves would seem to be due to a lessened breakdown of chlorophyll (or more probably the chlorophyll-protein complex in the chloroplast). Light effects on chlorophyll destruction have recently been discussed by Gabrielsen (1948). Potassium deficiency may then enhance the rate of breakdown or else delay the restoration of the complex. The second suggested cause of the improvement with shade is the greatly enhanced apical dominance under these conditions, which in barley suppressed tillering to a minimum. If it is assumed that the young leaf and stem primordia have the first call on any nutrient materials, especially those in short supply—and there is much evidence in favour of such a view—then increased apical dominance may well prevent the main shoots of the barley plant from being drained of potassium by the young tillers. Potassium concentration in the existing leaves of shaded plants may then never reach the same low level as in those exposed to full light, and their protein may therefore be maintained more effectively. As a further result of the leaves remaining functional for a longer time, together with the fact that new tillers are not produced to act as 'sinks', carbohydrate would be conserved, thus offsetting any adverse effect of the reduction in light on assimilation rate. In a plant such as bracken the absence of aerial shoots would at first sight make such an explanation appear rather dubious, but there is evidence that the leaves themselves control the expansion of new leaf buds by a process very much akin to, if not identical with, apical

dominance (Albaum, 1938). The numbers of leaves produced in the bracken shading experiment (omitting the dead plants) tend to confirm this.

Total No. of leaves produced per plant

Full light	{ F.M.	.	.	.	25.8	(4 replicates)
	{ K deficient	.	.	.	22.5	(2 „)
Shaded	{ F.M.	.	.	.	17.5	(4 „)
	{ K deficient	.	.	.	15.5	(4 „)

The two explanations for the favourable interaction effects between shading and K deficiency are not mutually exclusive, and it is likely that they apply concurrently.

Internal water contents are also closely allied to toxicity symptoms and accumulation of certain ions. The relation of the internal concentrations of K, Na, P, and Ca and water content have been exhaustively considered by Richards and Shih (1940). Apart from actual osmotic effects of the ions carbohydrate content has been shown to be the main factor controlling succulence in the plant. It is presumed to act by affecting cell-wall extensibility and thickness as also by the accumulation of starch, &c., in the cell. In the present experiment the starch contents of the rhizome are the only carbohydrate data available. A highly significant negative correlation obtains between starch and water content if both are calculated on a total dry-weight basis. However, if the relative contents are calculated on the basis of starch-free dry weight this correlation is no longer significant; and it is clear that the correlation on total dry weights depends on the fact that a large proportion of the total dry weight actually represents starch. Thus there is no obvious inverse relation on a 'per cell' basis as there would be if starch had actually displaced water from the cell; on the other hand, changes due to any direct displacement could only be small and therefore difficult to detect, especially in view of the compensating changes in osmotic pressure which would be caused. The small residual correlation after elimination of the starch from the total dry weight may perhaps be evidence of a real displacement effect. There does appear to be some inverse relationship between these two factors, however, whether causal or not, though it must be complex in its interaction with treatments, e.g. it is also seen in the mean age effect of falling succulence (S₃ to F.H. 905 to 689 per cent. starch-free dry weight) and rising starch content (S₃ to F.H. 56 to 117 per cent. starch-free dry weight).

As was pointed out in the first part of this paper, low pH values have generally been regarded as a requisite for the successful growth of the bracken fern. Wetzel (1938) mentions this, but also draws attention to the contradictory evidence of de Litardière (1933). This author found bracken growing on calcareous marl containing 60 per cent. lime. The pH of the rhizosphere was found to be as high as 8.33. The present experiments serve to confirm these observations. No significant effects were obtained in a small sand-culture

experiment in which bracken was grown in solutions differing by as much as three pH units. The main experiment confirmed this result, although, of course, all effects there due to pH were completely confounded with those due to solution type. However, the fact that all the high nutritional treatments of the main experiment, i.e. those showing the extreme of pH changes in the nutrient solution, produced large and healthy plants (save for some Na toxicity effects in the A solution) is good confirmatory evidence. That the symptoms seen in the HAK₁ plants were not the results of high pH is clear from their absence in the HCK₁ series with almost equally high pH values. It is evident therefore that bracken is largely unaffected by the soil reaction and capable of successful growth in a wide range of pH conditions.

4. *Comparison between deficiency effects on bracken and those described previously on barley and flax.* Only a brief account of the similarities and dissimilarities between the phanerogams used for nutrient experiments and bracken can be given, in addition to those already pointed out above.

TABLE XIII

Comparison of Dry-weight Reduction by similar deficiencies of N, P, and K in Barley, Flax, and Bracken (expressed as percentage of non-deficient controls)

Treatment.	Bracken.	Barley.	Flax.
N ₃ ($\frac{1}{10}$ N) . . .	33.2 %	16.9 %*	— %†
P ₃ ($\frac{1}{10}$ P) . . .	30.0	34.3*	15.6†
AK ₃ ($\frac{1}{10}$ K) . . .	22.9	49.5§	50.1†
CK ₃ ($\frac{1}{10}$ K) . . .	34.5	43.2§	63.1†
MK ₃ ($\frac{1}{10}$ K) . . .	18.6	32.0	44.8†

* Gregory, 1949. † No data available.

‡ Richards, 1942.

§ Shih, 1934.

|| Montgomery, 1938.

TABLE XIV

Comparison of the Relative Effects of Equivalent Reductions in K and P Supply on Several Characteristics in Barley and Bracken (expressed as $\frac{1}{10}$ th K: $\frac{1}{10}$ th P)

Characteristic.	Barley.	Bracken.
Meristematic activity (bud or tiller numbers) . . .	2.3†	1.3
Leaf area . . .	1.8†	1.5
Total dry weight . . .	1.9* and ‡	1.5
Carbohydrate content . . .	0.68†	0.96
Leaf water content§ . . .	1.10†	1.08

* Gregory, 1949. † Gregory, 1937. ‡ Shih, 1934.

§ (In this comparison the barley P level was only reduced to $\frac{1}{10}$).

Many effects are, of course, well known and similarities are to be expected. Examples of this kind are the reductions of dry and fresh weights, leaf area, &c., under deficiency conditions. But even here the degree of reduction is often quite similar, in spite of the fact that these decreases are far from being proportional to the lowering of the nutrient level, e.g. Table XIII. It is

proposed therefore to refer to some of the less obvious effects, which indicate that the agreement holds also in some detail.

The net assimilation rates for barley and bracken have been plotted in Fig. 5 for similar nutrient conditions, and the general agreement is apparent. The N.A.R.s of the HK1 bracken plants fall well into the range of 0.4–0.6 g./dm.²/week noted by Heath and Gregory (1938) for different plants in different seasons. They are: A, 0.47; C, 0.68; M, 0.56 g./dm.²/week. The average for all nutrient treatments is of course lower: 0.395.

Considering the dry-weight distribution it is interesting to find that in bracken as well as in barley suffering from lack of N the proportion of root to top is much increased.

The increase in starch content of the rhizome under P deficiency agrees well with the finding of Russell (1938), who noted increased contents of soluble carbohydrate in P-starved barley leaves (*N.B.* The only known nutrient treatment which will induce starch formation in the leaves of this plant). In N-deficient plants quantitatively similar increases in carbohydrate have also been found (Gregory and Baptiste, 1936).

Water contents are particularly instructive as here the large solution effects are paralleled in the two plants also. With K deficiency (K₃) water contents are increased in the A solution and lowered in the C and M.

The relative importance of similar reductions to both plants of P and K (to $\frac{1}{9}$ or $\frac{1}{10}$ th) is very much the same, as is seen from the similar yield ratios obtained in the two treatments (Table XIV). Divergencies exist, of course, such as the effect noted above of phosphorus supply in potassium deficiency in the A solution, but they appear unimportant relative to the general similarity of effects. It is clear then that the nutrient effects described and measured for barley and flax in the past apply in the main equally well to the cryptogam investigated.

CONCLUSIONS

The conclusions from these experiments can be divided into two groups, those of theoretical interest and those of practical importance. *Theoretically*, the above experiments are of value in confirming the general validity of the nutritional experiments carried out at this Institute. Their generality is much enhanced by the close similarity between the responses of bracken, barley, and flax to deficiencies of N, P, and K in widely differing basal solutions.

New observations of general interest are the probable NH₄ toxicity under P starvation in the M solution and the marked improvements due to shading in high sodium solutions with minimal K supply.

From a *practical* point of view, no new methods for the eradication of bracken have emerged from these investigations, indeed none were anticipated when the experiments were begun.

Heavy application of common salt may possibly assist in reducing the vigour of bracken, without at the same time permanently contaminating the soil, but the effect is unlikely to be lethal or a substitute for other methods such

as cutting (cf. also Angst (1925-8), who found bracken growing by the sea-shore).

It has been shown that bracken is at least as sensitive to lack of P as of K, if not more so (comparison between HK₃ and LK₁): and that the high K contents sometimes found in nature must represent luxury absorption.

Finally, the bracken fern has been shown to be largely insensitive to the reaction of the external solution, and is able to grow successfully over a wide range of soil pH, provided nutrient and other conditions are satisfactory.

ACKNOWLEDGEMENTS

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On the Megaspores of Four Species of *Lepidostrobus*

BY

W. G. CHALONER

(*University of Reading*)

With Plate VIII and twenty-three Figures in the Text

ABSTRACT

This paper is an account of the megaspores borne by four species of Carboniferous Lycopod cones, referred to the genus *Lepidostrobus*. The megaspores (assigned to the genus *Triletes*) have been known isolated for more than twenty years, but the identities of their parent cone species have not previously been established. This study suggests the limits of variation that can reasonably be allowed in a 'spore species'. Three of the cone species are emended in the light of their spore content, and a new species (*Lepidostrobus allantonensis*) is described. Using the megaspores as a diagnostic cone character, it has proved possible to correlate a species of isolated cone (*Lepidostrobus dubius* Binney) with the tree which bore it (*Lepidodendron simile* Kidst.). A cone previously attributed on doubtful evidence to *Bothrodendron* is shown to bear megaspores indistinguishable from those of a species reliably attributed to *Lepidodendron*, suggesting that the attribution to *Bothrodendron* is incorrect.

Preservation and Techniques

THE cones are in two states of preservation: ordinary compressions on carbonaceous shale and ironstone; and petrified, partially compressed specimens in clay-ironstone nodules. The type-specimens of *Lepidostrobus dubius* and *L. russelianus* and the large specimens of cones attached to *Lepidodendron simile* shoots are all in the former condition. The only two hermaphrodite specimens of *Lepidostrobus dubius* are in the latter condition. In the case of the compressions, the sporophyll laminas are sometimes only poorly preserved (being represented by carbonaceous streaks at the margin of the cone): they may even be missing entirely (as in the specimen figured by Binney, 1871, Pl. IX, Fig. 3, here assigned to *L. dubius*). In this case the cone consists only of the flattened axis, with aggregates of megaspores, representing the sporangia, arranged on either side of it. The sporophyll laminas may either have rotted away during fossilization or have been rubbed or broken off at an earlier stage.

Spores were removed from the cones by the use of celloidin peels, a mounted needle, or 'cellotape' pulls. In the latter technique (which was found particularly useful in large museum specimens rigidly mounted in an upright position) the cellotape was pressed against the fossil, pulled away, and the adhering fragments freed from the tape with benzene. The megaspores were macerated with sodium chlorate in nitric acid, followed by dilute ammonia. Some were

mounted dry and examined by reflected light (as recommended by Schopf, 1938; Dijkstra, 1946; and others), and some macerated until transparent, mounted in balsam, and examined by transmitted light (as recommended by Arnold, 1950). The latter method was found to show greater detail of wall structure and to be generally more satisfactory.

In all cases where microspores were obtained in any quantity, a minute fragment of cone-material was found to give large numbers of spores on maceration: these were centrifuged and mounted in balsam or glycerine jelly. Both megaspores and microspores in the clay-ironstone 'petrifications' were found to require only treatment with hydrochloric acid (followed by hydrofluoric acid in the case of the megaspores) to free them from both calcite and siliceous material in the matrix. The megaspores in this case, being unflattened and consequently unsuitable for mounting in balsam, were mounted 'dry'.

LEPIDOSTROBUS DUBIUS BINNEY 1871

1865. *Flemingites gracilis* Carruthers (Pl. 12, Fig. A).

1871. *Lepidostrobus dubius* Binney (Pl. IX, Fig. 3).

1871. *Lepidostrobus russelianus* Binney (Part: Pl. IX, fig. 2 only).

1938. *Sigillariostrobus sphenophylloides* Leclercq (Pl. I, Figs. 1-4).

Emended diagnosis

Cone cylindrical, or tapering slightly, 10 to 25 cm. long; body of the cone (excluding the sporophyll laminas) from 12 to 18 mm. wide, tapering rapidly in the region of the cone apex to a rather obtuse point. Cone axis 1.5 to 2.5 mm. diameter. Sporophylls arranged in whorls or a low-angle spiral; distance between successive whorls or turns of the spiral, 1.5 to 2.5 mm. Sporangia 5 to 8 mm. long (measured radially to the cone) and typically 2 mm. high (measured parallel to the cone axis). Sporangia in the immediate vicinity of the cone apex considerably smaller, and probably abortive. Sporophyll laminas turned towards the cone apex, and fairly closely appressed, or divergent from the cone at an angle of up to 45° from it: narrow, lanceolate, up to 1.5 mm. wide at the base, with a single median vein. Cone either containing megasporangia throughout, or megasporangia in the proximal part and microsporangia distally.

Megaspores belonging to *Triletes horridus* Zerndt (sensu Dijkstra 1946). Spheroidal, flattened along the polar diameter, with an apical prominence formed by an expansion of the contact faces. Mean diameter 1,062 μ (range 530-1,580 μ , for 419 spores from 21 cones). Triradiate ridges obscured by apical prominence. Arcuate ridges well developed. Height of apical prominence typically 500 μ (range 450-620 μ). Wall covered with spines, except for the apical prominence: spines typically 100 μ long, ranging up to 180 μ . Broad at the base, tapering to a fine point: crowded and shortest in the region of the arcuate ridges—longer, and more widely spaced on the distal surface of the spore. Between the spines, small elongated excrescences sometimes present,

blunt or pointed, up to 25 μ long, but typically 20 μ or less. These excrescences also sometimes present on the apical prominence. Spore wall finely granular, but the spines of clear homogeneous cuticular material. Wall typically 20 μ thick, range 13–26 μ . Flaps of the apical prominence rather thicker. Megasporeangia usually containing 6 tetrads (24 spores).

Microspores belonging to the spore genus *Lycospora* Schopf, Wilson and Bentall (1944). Sub-triangular in outline, mean diameter 19 μ , range 18–21 μ (mean of 100 spores from two cones). Triradiate ridges reaching almost to spore margin. Arcuate ridges developed to form a slight zonal flange. Distal surface decorated with numerous low papillae. Contact faces smooth. Microsporangia containing many thousands of spores.

Type specimen. The cone figured by Binney, 1871 (Pl. IX, Fig. 3), No. 472 a in the Sedgwick Museum, Cambridge, England. (From the Blackband Ironstone, Airdrie, Lanark, Scotland: *modiolaris* zone, Westphalian B).

Lepidostrobus dubius forma *megalphorus* (forma nov.)

Cone as described above, but containing only megaspores, which occupy the cone to the apex.

Type specimen. No. V30571, British Museum, Natural History. (From 'The Coal Measures', locality unknown.)

Lepidostrobus dubius forma *hermaphroditus* (forma nov.)

Cone as described above, containing megaspores in the proximal part and microspores in the distal part.

Type specimen. No. V1244, British Museum, Natural History. From Coseley, nr. Dudley, Staffordshire. 'Middle Coal Measures.'

Time range of L. dubius

This species occurs in the Coal Measures, from the *lenisulcata* zone to the upper part of the *modiolaris* zone (Westphalian A and B). (Correlation given by Trueman, 1946).

Distribution. From the Central Coalfield of Scotland and the coalfields of Yorkshire, Lancashire, and Staffordshire.

Other specimens of L. dubius

Forma hermaphroditus. No. T. 468 Geology Dept., University College, Exeter, Devon. Horizon unknown, Lancashire coalfield.

Specimens of unknown forma. No. 471 a. Sedgwick Museum, Cambridge. Figured by Binney, 1871, Pl. IX; Fig. 2. From the Blackband Ironstone, Airdrie, Lanarkshire, Scotland. (*Modiolaris* zone, Westphalian B). Nos. 1587, 1588. Kidston Collection, Geological Survey & Museum, London. From 12 ft. below the Ell Coal, nr. Kilmarnock, Ayrshire, Scotland. (*Communis* zone, Westphalian A.) No. 2783, Kidston Collection, Geological Survey & Museum, London. Figured by Leclercq as '*Sigillariostrobus sphenophylloides*'. From the Better Bed Coal, Yorkshire. (Base of the *communis* zone, Westphalian A.) No. 13733, British Museum, Natural History, London.

From the lower Main Shales, Gosforth, nr. Newcastle (*Modiolaris* zone, Westphalian A-B.) No. Z74. Botany Dept. Collection, Royal Holloway College (University of London), Englefield Green, Surrey, England. From the Coal Measures, locality unknown. See also below, for specimens associated with *Lepidodendron simile*.

Discussion

(a) *The name.* Binney first used the name *Lepidostrobus dubius* in 1871, applied to a cone from the Airdrie Blackband. Carruthers had used the name *Flemingites gracilis* 6 years earlier to describe a cone of exactly the same type from the same locality, believing the megaspores in the cone to be two rows of rounded sporangia on the upper surface of each sporophyll. He considered that his specimen differed in this respect from *Lepidostrobus* (with only one sporangium per sporophyll), and so erected the genus *Flemingites* on the basis of this mis-interpreted specimen. I have obtained megaspores agreeing with *Triletes horridus* from the type specimens of both *Lepidostrobus dubius* and *Flemingites gracilis*; and the two cones themselves agree in all essential features. The combination *Lepidostrobus gracilis*, which would be the proper designation of the species, had already been used for an entirely different cone by Newberry (1853). Binney's name, *L. dubius*, is therefore the first valid name for this species.

(b) *Taxonomic treatment.* Since *Lepidostrobus* cones evidently tended to break up before fossilization, pieces of cones are much more common than whole ones. Any realistic taxonomic treatment of these fossils must therefore take into account these cone fragments. Specimens of *L. dubius* are known in which only megaspores occur, occupying the cone right up to its apex; and others, which are structurally the same and which bear identical megaspores in their basal parts, have microspores in the apical region. Clearly, fragments from the basal part of these two cone types would be indistinguishable. There seems no justification for separating megaspore-bearing and hermaphrodite cones into two species on that basis alone. I have therefore adopted the course followed by Schopf (1941), and used two names of the status of formae to distinguish the two types of cone. Cones consisting of a megaspore-bearing fragment, truncated at both ends, can be assigned to the species *Lepidostrobus dubius*, but their forma cannot be specified. The type specimen of the species is a fragment of this kind.

(c) *The cones.* Owing to the nature of the preservation of the specimens of this species, relatively little of the structure of the cone itself can be recognized compared with cone petrifications. The present emended diagnosis is therefore dependent to a considerable extent on the characters of the spores. In this respect the definition of the species is analogous to that of *Lepidostrobus braidwoodensis* Arnold (1938), in which the cone has no distinctive character in itself, but rather depends for its definition on the characters of the well-preserved megaspores. I have made preparations of megaspores from all the cones cited under *L. dubius*, and data from all of them has been used in

drawing up the diagnosis of the species. Likewise, preparations of the microspores have been made from the two specimens of the forma *hermaphroditus*. Cones resembling *L. dubius* superficially but from which no spores have been obtained cannot be correctly assigned to *L. dubius* but should be placed in *L. variabilis* L. & H. (see p. 282 below).



TEXT-FIG. 1. *Triletes subpilosus* (Ibrahim) S, W, and B. $\times 50$. Determined by Dr. Dijkstra. Fig. 1A, $\times 150$, showing spines in the apical region, and 1B, $\times 150$, showing spines in the distal region. (Brit. Mus. Nat. Hist. V27918a.)

TEXT-FIG. 2. *Triletes horridus* Zerndt, $\times 50$. Determined by Dr. Dijkstra. Fig. 2A, part of the spore coat, $\times 150$. (Brit. Mus. Nat. Hist. V27914a.)

(d) *The megaspores.* Dijkstra (1946) recognizes only three species of spiny megaspores within the Lagenicula group of *Triletes*: *T. subpilosus*, *T. horridus*, and *T. crassiaculeatus*. He has included in these species several others of Zerndt and other Continental workers. Although Schopf (1949) has criticized Dijkstra for his broad interpretation of megaspore species, I have accepted Dijkstra's interpretation of these three species as the only attempt at a critical integration of this rather confused group. Dr. Dijkstra has kindly sent me specimens of all three of these species and also of his *Triletes subpilosus* forma *major*.

The main distinction between *Triletes subpilosus* (Text-fig. 1) and *Triletes horridus* (Text-fig. 2) is that in the former the spiny covering of the spore is

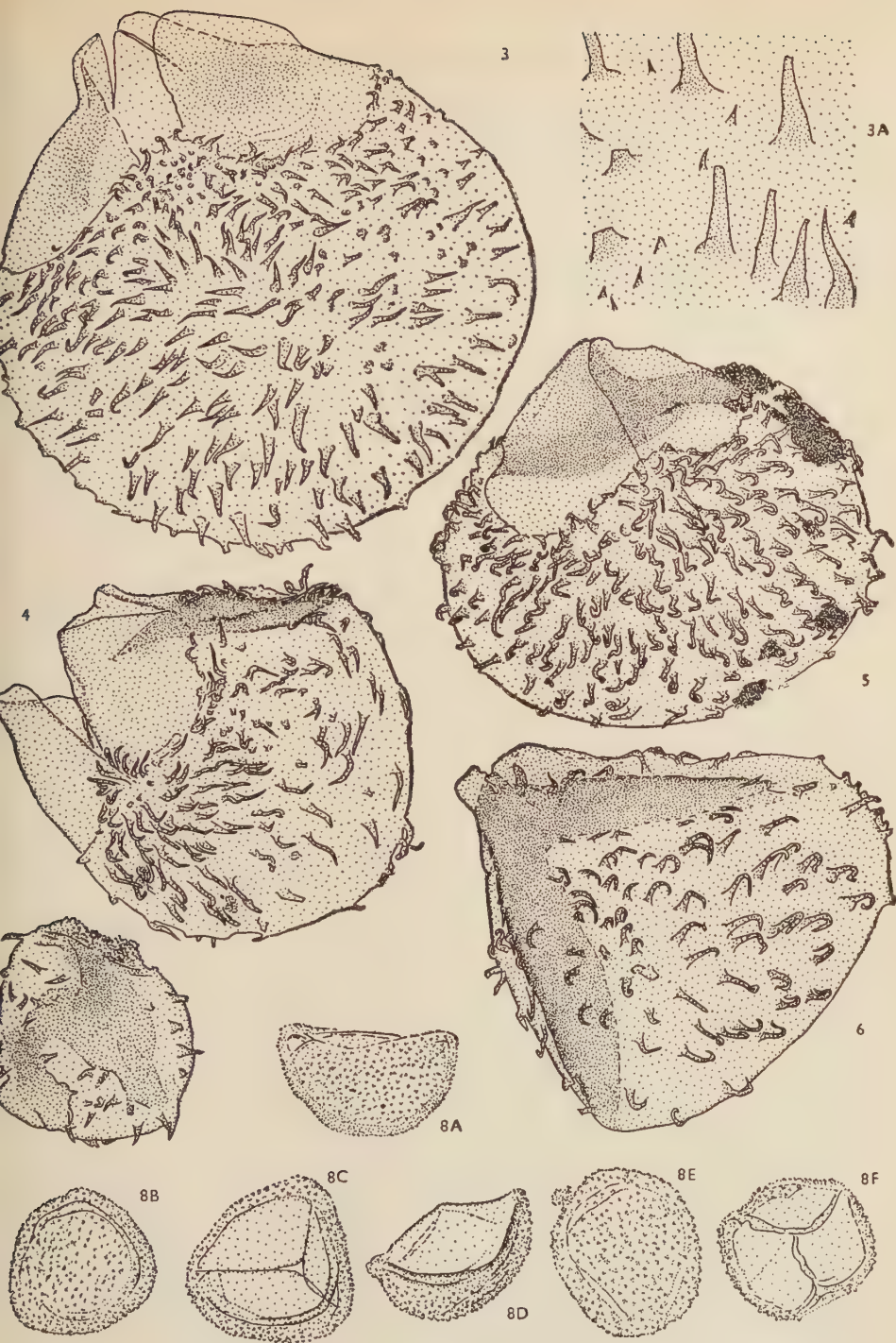
much denser, and the spines are more or less parallel sided and are only slightly expanded at the base. In *Triletes horridus* the spiny covering is relatively less dense, and the spines taper throughout their length to a sharp point and have a swollen trumpet-shaped base.

The size of the megaspores of *Lepidostrobos dubius* as cited above rather exceeds that given by Dijkstra (1946) and Arnold (1950) for *Triletes horridus*. Arnold, who gives the larger range, records the maximum of 1,300 μ , whereas *L. dubius* spores range up to 1,580 μ . This small extension in the size range alone does not seem sufficient basis for excluding the megaspores of *L. dubius* from *Triletes horridus* since they otherwise agree so closely in general shape and structure, the type and size of the spines and subsidiary excrescences, and the spore-wall texture. Megaspores from some of the cones assigned to *Lepidostrobos dubius*, including those figured by Binney (1871) and Leclercq (1938), are shown in Text-figs. 3-7.

In all the histograms of megaspores (and of microspores) in this paper, only a single measurement of each spore is used—namely, the maximum diameter of the flattened spore, excluding the apical prominence. Arnold (1950) discusses the difficulties of making precisely orientated measurements of spores of the Lagenicula type. All the spores obtained from the cones cited under *L. dubius* were used in compiling the histogram of Text-fig. 9. Since different numbers of megaspores were obtained from the different cones, the histograms in no way represent a compilation of equal numbers of spores from each cone. Rather they indicate that there is a distribution about a mean value for megaspore size, and that there are relatively small numbers of spores in the extreme size ranges. The size units along the abscissae of the histograms in Text-figs. 9 and 18 represent units on the scale of the micrometer used in the spore measurement, which have been converted directly into μ , and left in these groups for convenience.

One of the most variable features of the megaspore decoration in *Lepidostrobos dubius* is the presence and abundance of the small excrescences between the main spines (Text-fig. 3A). These are described and figured by Arnold (1950, pl. X, Fig. 4) on *Triletes horridus*, and mentioned by Zerndt (1934, p. 26). Arnold records these excrescences as occurring in about 20 per cent. of the *T. horridus* spores that he examined, and illustrates them in his photograph of a transparent preparation. Within a single cone, the variation in density of these excrescences is considerably less than the range for all the cones of *Lepidostrobos dubius* that I have examined. For example, the excrescences were very abundant on all the megaspores from the specimen in the Royal Holloway College Collection, No. Z74; but the spores from one of the *Lepidostrobos dubius* specimens associated with *Lepidodendron simile* (see below) varied from being entirely without these excrescences to having only a few of them. This bears out Arnold's suggestion that the variation that he observed in isolated spores is not of specific significance.

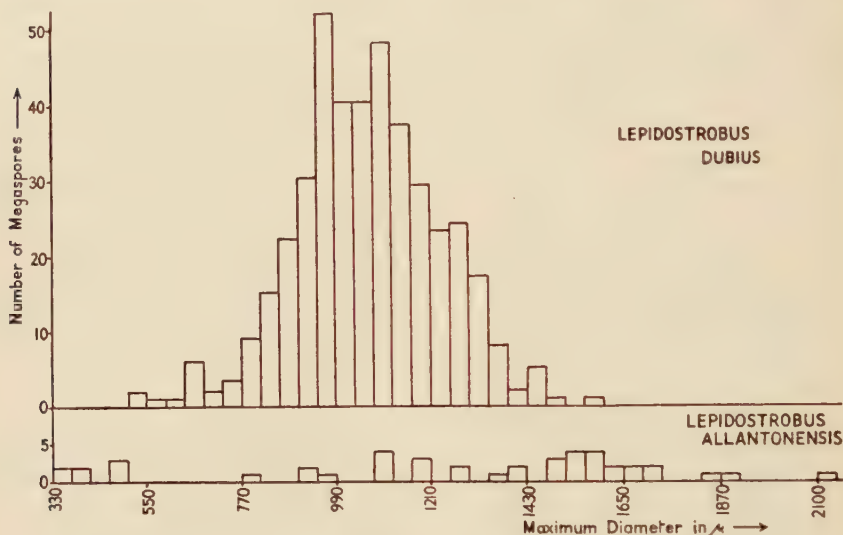
A detail observed on some of the spores of *Lepidostrobos dubius* not mentioned by Arnold in his description of *Triletes horridus* is the presence of



TEXT-FIGS. 3-7. *Triletes horridus*, from *Lepidostrobos dubius*, all $\times 50$. Fig. 3, from the specimen described by Leclercq as '*Sigillariostrobus sphenophylloides*'. Fig. 3A, part of the spore, $\times 150$ (2783s, Kidston Collection). Fig. 4, from the type specimen of *L. dubius* (472b, Sedgwick Museum). Fig. 5, from the type of *Flemingites gracilis* (V6041, j., Brit. Mus. Nat. Hist.). Fig. 6, from the cone shown in Pl. VIII, Fig. 1. (L 10878a, Manchester Museum). Fig. 7, from the apex of the type of *L. dubius*, forma *megalophorus* (V30571g, Brit. Mus. Nat. Hist.).

TEXT-FIGS. 8a-f. Microspores of *L. dubius*, $\times 1,000$. (a-c, V1244d, Brit. Mus. Nat. Hist.; d-f, T468w, Geology Dept. Collection, University College, Exeter.)

globules of cuticular material matching the colour and texture of the spines, occurring in aggregates or scattered singly over the surface of the spore between the spines (cf. Text-fig. 7, top right margin and similar bodies on the *L. russelianus* spores, Text-fig. 13A). The globules vary considerably in size, up to $30\ \mu$ diameter, and appear to be nearly perfect spheres. They are present on only quite a small proportion of the spores. It seems possible that they may represent the fossilized contents of the sporangium in the form of the residue



TEXT-FIG. 9. Histogram of megaspores of *Lepidostrobus dubius* (above) and *L. allantonensis* (below). The former based on 21 cones and the latter on 6 cones. The measurements along the abscissa are of the maximum diameter of each spore, excluding the apical prominence.

of the tapetum. I do not regard them as forming part of the characteristic spore decoration. I have also observed only a few of the spines to have the globular tips described by Arnold (1950). It seems unlikely that they are present on all the spines when in the intact condition, as he has suggested, since they are not common even on the spores dissolved out of the calcite infilling of the cones in the clay-ironstone nodules.

(e) *The microspores.* The microspores obtained from the only two known hermaphrodite specimens of *Lepidostrobus dubius* show a remarkably close agreement in size and structure (cf. histograms based on 50 spores from each of these two specimens in Text-figs. 19A and B). Each measurement of a spore on the histogram is its maximum diameter, which minimizes the distortion of the histogram by badly crumpled spores. Since the preservation of these two hermaphrodite *L. dubius* cones is such that relatively little crushing of the microspores has occurred, it is possible to focus on the proximal and distal surfaces of the spore independently. Further, spores occasionally come to rest on edge in the mounting medium, showing the spores in profile. Text-figs.

8 A-F show the proximal (C, F) distal (B, E) and profile (A, D) views of spores from the two hermaphrodite cones. In the figures an attempt has been made to portray the punctate sculpturing on the distal surface of the spore, in contrast to the undecorated proximal surface.

The microspores of *L. dubius* do not appear to fit any of the previously described species of *Lycospora*, although their structure is clearly within the limits of this isolated spore-genus as defined by Schopf, Wilson, and Bentall (1944). They are rather similar to *Lycospora micropapillatus* S., W. & B. (1944), but lie outside the size range of this species, as quoted by Knox (1950); viz., $15\text{ }\mu\text{--}16\text{ }\mu$ for *Lycospora micropapillatus* and $18\text{ }\mu\text{--}21\text{ }\mu$ for *Lepidostrobus dubius* microspores. The microspores from this cone species are also very close to *Lycospora brevijuga* Kosanke (1950) in structure, but have an average size only about two-thirds that of this species.

(f) *Comparison with other species of Lepidostrobus.* The only previously described species of *Lepidostrobus* that is at all similar to *L. dubius* is *L. veltheimianus*, a petrified cone from the Pettycur Limestone (Lower Carboniferous) of Scotland, attributed to *Lepidodendron veltheimianum* by Scott (1920) on the basis of association. Although these petrified cones are hermaphrodite and have spinose megaspores (described by Williamson, 1872), these are considerably smaller (mean diameter $500\text{ }\mu$) than those of *Lepidostrobus dubius* (mean diameter $1076\text{ }\mu$). Also, the spines in *Lepidostrobus veltheimianus* spores appear to be of more or less uniform width throughout their length, with blunt or swollen apices (Williamson, 1872, Pl. XLIV, Fig. 27). The petrified cones themselves are also no more than 1 cm. diameter, as against 1.5 cm. or more for *L. dubius*. Although the comparison between the two species is limited to a considerable extent by their different states of preservation, I believe that the differences cited above are sufficient basis for specific separation.

(g) '*Sigillariostrobus sphenophylloides*' Leclercq. Having examined the type specimen of this species, and a number of preparations made from it (both Leclercq's and my own), I believe this cone to be identical with *Lepidostrobus dubius* Binney (emend.). The form and size of the cone are typical for this species. The lower sporophylls have been shed, as is the case in the specimen of *L. dubius* (Binney 1871, Pl. IX, Fig. 2), and the sporophylls of Leclercq's cone are arranged in whorls, and not in spirals, as in most specimens of *L. dubius*: nevertheless, other examples of *L. dubius* with whorled appendages are also known (cf. Pl. VIII, Fig. 3).

The megaspores obtained from '*Sigillariostrobus sphenophylloides*' (one of which is shown in Text-fig. 3) are *Triletes horridus*. Leclercq (1938, p. 165) has, however, compared them to Zerndt's type 14 (*Triletes mamillarius* Zerndt), which differs in a number of respects from *T. horridus* and in fact belongs to a different group within *Triletes* (the Aphanozonati, which lack the apical prominence of *T. horridus* and related types). Schopf (1941, p. 30) has already suggested, from an examination of Leclercq's figures alone, that the spores of '*Sigillariostrobus sphenophylloides*' agree more closely with

Triletes (*Lagenicula*) *kidstoni* Zerndt than with *T. mamillarius*. (*Lagenicula kidstoni* Zerndt is included by Dijkstra, 1946, in *Triletes horridus*.) The spore shown in Text-fig. 3 and all other megaspore preparations from Leclercq's cone show the long spines and apical prominence which characterize *Triletes horridus* and distinguish it from *T. mamillarius* Zerndt. Part of the apical prominence is in fact visible in Leclercq's Fig. 10, Pl. IV, at the top right-hand side, the outermost part having broken away.

I do not believe that there is any clear evidence as to whether or not this cone originally bore microspores in the apical region (see discussion on pp. 288–90). The attribution of Leclercq's cone to *Sigillariostrobus* was on the basis of the whorled appendages and association with *Sigillaria*. For the reasons stated below (pp. 287–8) I do not accept the whorled arrangement of the sporophylls on its own as a valid diagnostic of *Sigillaria* cones. The association with *Sigillaria* is not very close, for there are no fragments on the slab bearing the type specimen. Several species of both *Lepidodendron* and *Sigillaria* are recorded from this horizon. In the absence of evidence as to its true microspore contents, this cone must simply be placed in *Lepidostrobus dubius* and cannot be assigned to one or other of the formae.

(h) *The connexion of Lepidostrobus dubius with Lepidodendron simile*. Cones agreeing with *Lepidostrobus dubius* occur in close association and organic connexion with *Lepidodendron simile* in three specimens. Two of these are in the Manchester Museum (Nos. L10878 and L12432) and one in the British Museum, Natural History, London (No. V12024). All three specimens are large slabs of shale, mounted rigidly in an upright position. Of the 25 cones present on the 3 specimens, 3 are in undoubted organic connexion (2 of these being shown in Pl. VIII, Fig. 1). Although I was unable to examine the cones as closely as I would have wished, I took 'cellotape' peels from the 25 cones, and 10 of these yielded *Triletes horridus*, all agreeing perfectly with those obtained from isolated *Lepidostrobus dubius*. (One of these spores, obtained from a cone shown in Pl. VIII, Fig. 1, is shown in Text-fig. 6). These cones, if found isolated, would be correctly assigned to *Lepidostrobus dubius*, on the basis of their external form and the character of their megaspores.

Only 1 of the 25 cones yielded microspores, and these, although of the *Lycospora* type, and of uniform character, were so few in number that I cannot regard them as being more than evidence of their close association with these *Lepidostrobus dubius* cones. Since the 'cellotape' peels bring away only very small fragments of the fossil, I do not regard either the absence of microspores, or the failure to obtain megaspores from 15 of the 25 cones, as being of great significance. As there is therefore no conclusive evidence about their microspores, these cones cannot be assigned to one or other of the two forms of *Lepidostrobus dubius* cones.

All three of these large *Lepidodendron simile* specimens had been labelled *Lepidodendron lycopodioides* Sternb. The group of *Lepidodendron* species characterized by rather small but persistent leaves, which includes most specimens previously assigned to *Lepidodendron lycopodioides* Sternb., has

recently been revised by Nemejc (1947). He recognizes three species within this group: *Lepidodendron ophiurus* Bgt., *L. simile* Kidst., and *L. acutum* Presl. He rejects the old name *L. lycopodioides* Sternb. as being a confusion of more than one type, and places most of the specimens previously attributed to it in *L. simile* Kidst. He regards this species as being characterized by the relatively narrow lanceolate leaves, curved in a sigmoid manner and joining the shoot in an acute angle. In *Lepidodendron ophiurus* the leaves are similar, but join the shoot nearly at right angles and have a simple curvature. The leaves of *L. acutum* Presl. are also similar, but become broader and longer on the older branches.

The leafy shoots of all three of these large specimens associated with *Lepidostrobus dubius* cones appear to fit *Lepidodendron simile* Kidst. as defined by Nemejc (1947). Leafy shoots of one of these specimens are shown in Pl. VIII, Fig. 1. The leaves are up to 2 cm. long and 2 mm. broad, and they show the sigmoid curvature that Nemejc regards as characteristic of this species. The leaves appear to be retained by the shoots until the branches are up to 3 cm. diameter, while larger stems lacking leaves are partially decorticated and resemble the 'Knorria condition'. It seems likely that breakdown of the outer cortex sometimes occurred immediately after, if not simultaneously with, the shedding of the leaves of this species.

Nemejc (1947) describes the cones of *Lepidodendron simile* Kidst. as being long and cylindrical, 1.5 cm. in diameter, with an axis 2 mm. thick. Clearly this description could include cones agreeing with *Lepidostrobus dubius*. Several authors figure such cones attached to *Lepidodendron simile* (e.g. Bureau, 1914, Pl. 34; Nathorst, 1894, Pl. XVI, Fig. 11), but without the evidence of the contained spores, such cones cannot be assigned to *Lepidostrobus dubius*.

The horizon of only two of the three large *Lepidodendron simile* specimens is known, and is that of the Upper Foot Mine (*lenisulcata* zone: Lancashire coalfield). This lies within the Westphalian A, and so is also within the time range of *Lepidostrobus dubius* as known from isolated specimens.

LEPIDOSTROBUS ALLANTONENSIS SP. NOV.

Diagnosis

Cone cylindrical, 11 cm. or more in length, the body of the cone (excluding the sporophyll laminas) 12–16 mm. diameter. Cone axis 1.5 to 2.5 mm. diameter. Sporophylls arranged in whorls or in a low-angle spiral: distance between successive whorls or turns of the spiral, up to 1.5 mm. Sporangia, up to 7 mm. long, and 1.5 mm. high.

Megaspores belonging to *Triletes crassiaculeatus* Zerndt (sensu Dijkstra, 1946). Spheroidal, flattened along the polar diameter, with an apical prominence formed by an expansion of the contact faces. Mean diameter $1,383 \mu$ (range for 43 spores, 350 – $2,120 \mu$). Apical prominence typically 590μ high (range 450 – 750μ). Wall covered with spines, except for the apical promi-

nence. Spines typically 200 μ long (ranging up to 305 μ). Broad, often fluted at the base, tapering to a fine point; crowded and shortest in the region of the arcuate ridges; longer and more widely spaced on the distal surface of the spore. Between the main spines small subsidiary spines usually present; narrow, tapering to a fine point, typically 35 μ long, but ranging up to 70 μ . Many very small blunt excrescences usually present on the apical prominence. Spore wall finely granular, but the spines of clear homogeneous cuticular material. Wall typically 20 μ thick, ranging up to 32 μ . Flaps of the apical prominence rather thicker.

Type Specimen (Pl. VIII, Fig. 2). No. 2972 Kidston Collection, Geological Survey Museum, London. From the Whiteadder Water, right bank, 1 mile east of Allanton Bridge, Berwickshire, Scotland. Calciferous Sandstone Series, Dinantian.

Range. This species is recorded only from the Allanton locality, in the Calciferous Sandstone.

Other Specimens. Nos. 2752, 2753, 2754, 2761, 2764, Kidston Collection, Geological Survey Museum, London. All are from the right bank of the Whiteadder Water, within a mile of Allanton, Berwickshire.

The name is derived from the type locality.

Discussion

Megaspores have been obtained from all the cones assigned to this species, but the microspores are unknown. Three of the megaspores are shown in Text-figs. 10–12. As with *Lepidostrobus dubius*, *L. allantonensis* is defined largely in terms of its megaspore characters. These two species can in fact only be distinguished on the basis of their contained spores. The megaspores of *L. allantonensis* agree well with the data for *Triletes crassiaculeatus* given by Dijkstra (1946), and with the specimens of that species which he has kindly sent me for comparison. The table below shows comparative data for *Triletes horridus* and *T. crassiaculeatus* given by Dijkstra (1946) and for *Lepidostrobus dubius* and *L. allantonensis* megaspores. (Where the ranges given by Dijkstra are exceeded by those of Arnold, 1950, the larger range is given, so giving the broadest limits of the species. The figures in brackets after the mean or 'typical' values are the ranges. All measurements are expressed in μ .)

	<i>Triletes horridus</i>	<i>Lepidostrobus dubius megaspores</i>	<i>Triletes crassiaculeatus</i>	<i>Lepidostrobus allantonensis megaspores</i>
Spore size . . .	850 (570–1000)	1062 (530–1580)	(800–1900)	1383 (350–2120)
Height of apical pro- minence . . .	430 (350–600)	500 (450–620)	(350–800)	590 (450–750)
Spine length . . .	(100–150)	100 (to 180)	(84–360)	200 (to 305)
Subsidiary spines or excrescences	(10–20)	20 (to 25)	(to 48)	35 (to 70)
Wall thickness . . .	20	20 (to 26)	to 48 (Zerndt 1937)	20 (to 32)

Only 43 megaspores were obtained from the 7 specimens of *L. allantonensis* and all of these were plotted in the histogram in Text-fig. 9. There is no suggestion of a mode in this population, but the range is clearly much greater than in *L. dubius*. Owing to this great size variation in a relatively small sample, the mean deviation of *L. allantonensis* megaspores in this population would necessarily be very great, and it is therefore superfluous to test for significance



TEXT-FIGS. 10–12. Megaspores from *Lepidostrobus allantonensis* sp. nov. Figs. 10, 11, from the same cone, $\times 50$. Fig. 10A, part of 10, $\times 150$, to show the subsidiary spines. Both from 2764a, Kidston Collection. Fig. 12, a megaspore from the type specimen (Pl. VIII, Fig. 2), $\times 25$.

of difference between this species and *L. dubius*, as this would be consequently very low.

The main distinction between fully developed spores of *L. allantonensis* and *L. dubius* is the larger size in all features of the former. The apical prominence, although not always as elongated as in the type figure of *Triletes crassiaculeatus* (Zerndt 1931, Pl. 9, fig. 28), is considerably larger in the fully developed spores of *L. allantonensis* than in *L. dubius* (cf. Text-fig. 10). The main spines on the distal face of the spores from the Lower Carboniferous cone species are nearly twice the maximum of those of *L. dubius*. The subsidiary spines, between the main ones, are larger and often form a more dense covering than in *L. dubius*. There is thus close agreement between the largest of the

L. allantonensis megaspores and *Triletes crassiaculeatus*, including the dimensions of the two sets of spines, the apical prominence, and the spores themselves. But as Arnold (1950, p. 82) has pointed out, the differences between *T. crassiaculeatus* and *T. horridus* 'are only those of degree'. The distinction between *L. dubius* and *L. allantonensis* is apparent only in the fully developed spores: but in both species there are in an average cone a few small (? abortive) spores as well as the larger ones (see histogram, Text-fig. 9, and Text-fig. 11). Although for the reasons given below I do not believe these small spores to be immature, it must be recognized that it might not be possible to distinguish a specimen of *L. allantonensis* containing only one or two of these small abnormally developed spores, from a corresponding *L. dubius* cone.

Globular swellings are present on some of the spines of the *L. allantonensis* megaspores, although none are present on those of the *Triletes crassiaculeatus* determined by Dijkstra. I do not regard this as of great consequence, since they could so easily have been knocked off the spines of spores obtained from coal macerations. Adhering to some of the *L. allantonensis* spores there are also globules, similar to those described for *L. dubius* megaspores.

Dijkstra (1952) records *T. crassiaculeatus* as occurring only in the Namurian and Dinantian, from Poland and Turkey. There is thus agreement in age also between the spores in *L. allantonensis* cone and the various records of the same type of spores (*T. crassiaculeatus*) found in coals elsewhere.

Unfortunately the microspores of *L. allantonensis* are unknown; for they would offer added data for comparison with *L. dubius*. Even without them, there are two additional pieces of evidence which support the separation of these two species. Firstly, there is a big difference in age between the Calcareous Sandstone Series (with *L. allantonensis*) and the oldest specimens of *L. dubius* in the Coal Measures. Secondly, as shown above, *L. dubius* cones were borne on *Lepidodendron simile*. This species of *Lepidodendron* is only known from the Upper Carboniferous; and the only species of *Lepidodendron* that I know of, recorded from Allanton, is *L. nathorsti* Kidston. It seems at any rate plausible that *L. allantonensis* is the cone of *Lepidodendron nathorsti*, and very unlikely that it was borne by *Lepidodendron simile*. This difference in age and in probable parent *Lepidodendron* species, though not actual justification for separation, is added evidence to support the treatment of *L. allantonensis* and *L. dubius* as separate species.

Dijkstra (1952, p. 13) states that, in the opinion of Professor Jongmans and himself, *Triletes crassiaculeatus* is the megaspore of *Lepidodendron acuminatum* Goepf. This correlation is based on very significant association at one locality. It seems quite possible that megaspores agreeing with *Triletes crassiaculeatus* were borne both by *Lepidodendron acuminatum* and *L. nathorsti* (in the latter case in cones of *Lepidostrobus allantonensis* type). It is shown below that another species of *Triletes* was also borne by two different cone species.

LEPIDOSTROBUS RUSSELIANUS BINNEY

1871. *Lepidostrobus russelianus* Binney, part (Pl. IX, Fig. 1 only).
 1871. *Lepidostrobus hibbertianus* Binney (Pl. X, fig. 2). (Also refigured in Scott, 1920, fig. 73.)
 1949. *Lepidostrobus* sp. Arnold (Pl. X, Fig. 2).

Emended Diagnosis

Cone cylindrical, or tapering slightly, up to 15 cm. long, with the body of the cone (excluding the sporophyll laminas) from 12 to 20 mm. wide. Cone tapering rapidly in the region of the cone apex to a rather obtuse point. Cone axis 1.5 to 2.5 mm. diameter. Sporophylls arranged in whorls, or a low-angle spiral; distance between successive whorls or turns of the spiral, 1.2–2.0 mm. Sporangia 5–8 mm. long (measured radially to the cone) and typically 2 mm. high. Sporangia in the vicinity of the cone apex considerably smaller, and probably abortive. Sporophyll laminas closely appressed to the cone, their apices turned towards the cone apex; narrow, probably lanceolate. Megasporeangia at the base of the cone, microsporeangia at the apex; or alternate zones of megasporeangia and microsporeangia, with ultimately microsporeangia apically and megasporeangia at the base.

Megaspores belonging to *Triletes rugosus* (Loose) Schopf; spheroidal, with an apical prominence. Mean diameter of the flattened spore 830 μ (range 350–1,230 μ). The apical prominence formed by expansions of the contact faces, typically 370 μ high (from apex to arcuate ridges), range 280–470 μ . Arcuate ridges well developed. Walls typically 13 μ thick, range 8–19 μ . Flaps forming the apical prominence considerably thicker. Spore wall finely granular; pits or hollows in the outer surface up to 15 μ diameter present on some spores. Aggregates of globules of cuticular material (up to 13 μ diameter) occasionally present, adhering to the spore wall.

Microspores belonging to *Lycospora* S. W. & B. When flattened, circular in outline; mean diameter 25 μ , range 20–34 μ . Triradiate ridges reaching to spore equator, which is slightly thickened to form a narrow zonal ridge, about a quarter of the spore radius in width. Wall decorated with small rounded papillae, covering only the distal surface.

Type specimen. The cone figured by Binney (1871, Pl. IX, Fig. 1), No. 32a in the Sedgwick Museum, Cambridge, England. From the Blackband Ironstone, Airdrie, Lanarkshire, Scotland (*modiolaris* zone, Westphalian B).

Time range. In Britain, from the *communis* zone (Westphalian A) to the ? *phillipsi* zone, Westphalian C. In the U.S.A., the 'late Westphalian B' (Arnold, 1949).

Distribution. The Central Coalfield of Scotland; the coalfields of Lancashire, Yorkshire, the Forest of Wyre (Worcestershire) and the Forest of Dean (Gloucestershire), England; also from Grand Ledge, Michigan, U.S.A.

Other specimens of this species. No. 473a, Sedgwick Museum, Cambridge. Figured by Binney, 1871, Pl. X, Fig. 2. The type specimen of '*Lepidostrobus*

hibbertianus' Binney. Believed to be from the Blackband Ironstone of Airdrie, Scotland (see below). No. 46a, Sedgwick Museum. From the Blackband Ironstone, Airdrie, Scotland. (*Modiolaris* zone, Westphalian B.) No. L7131, Manchester Museum. From Sparth, Rochdale, Lancashire. (*Communis* zone, Westphalian A.) No. K1, Geology Dept. Collection, Bedford College, University of London. From the Barnsley Bed, Barnsley, Yorkshire. (Base of the *similis-pulchra* zone, Westphalian B.) No. 5547, Kidston Collection, Geological Survey, London. Bayton Colliery, Forest of Wyre, Worcester. (? *phillipsi* zone, Westphalian C.)

All of the above contain both microspores and megaspores, excepting the specimens from the Manchester and Geological Survey museums, which contain megaspores only.

Discussion

(a) *The name.* In his monograph of 1871 Binney figured two specimens under the name of *Lepidostrobus russelianus*. One of these (Pl. IX, Fig. 1, Binney, 1871, now No. 32a in the Sedgwick Museum) contains *Triletes rugosus*, and I designate this the type of the species, as emended above (a spore from this cone is shown in Text-fig. 13). The other specimen figured under the same name by Binney (Pl. IX, Fig. 2, No. 472a in the Sedgwick Museum) contains only *Triletes horridus* spores, and so I have placed it in *L. dubius* as emended above. Either of these two cones figured by Binney would have had equal claim to have retained the name *Lepidostrobus russelianus*. But since the second of these two (No. 471a) agrees with *Lepidostrobus dubius* (of which Binney designated only one type specimen, containing *Triletes horridus* spores), it seems most satisfactory to place it in that species, leaving the first of the two specimens (No. 32) as the type of *Lepidostrobus russelianus* emend.

(b) *Taxonomic treatment.* There is evidence in four of the specimens of *Lepidostrobus russelianus* examined of both microspores and megaspores being borne in the same cone. The arrangement was either of microspores distally and megaspores proximally, or with an additional zone of each in the middle region, as in the type specimen (see below). There are no specimens in which there is any evidence to suggest that megaspores were borne throughout the cone. I have therefore placed all the cones under one specific name, whether microspores are present or not.

(c) *Cone structure.* Some specimens of *L. russelianus* have a whorled arrangement of the sporophylls (cf. Bedford College Collection, No. K1) and others a spiral phyllotaxy (cf. Sedgwick Museum, No. 46). As in *L. dubius*, I do not regard this as of any significance as a diagnostic feature.

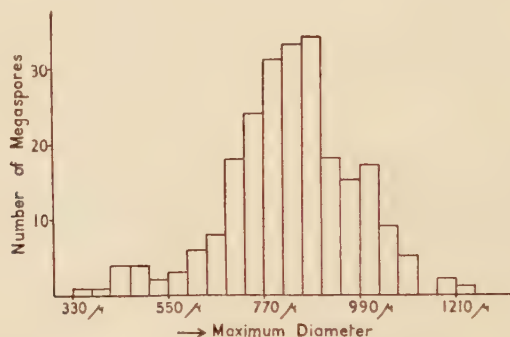
In Binney's figure of '*Lepidostrobus hibbertianus*' (1871, Pl. X, Fig. 2), here assigned to *L. russelianus*, he illustrates what are described as 'four of the rhomboidal scales or ends of the bracts from the upper part of the cone'. But examination of the specimen shows that these are in fact the ends of the microsporangia, forced into a rhomboidal shape by mutual pressure within the cone. As in most of the compressions, the actual sporophyll laminae are



TEXT-FIGS. 13-16. *Triletes rugosus* (Loose) Schopf, from *Lepidostrobus russelianus*, Binney emend, $\times 50$. Fig. 13, from the type specimen, lower megaspore zone. Fig. 13A, part of the same, $\times 150$, to show cuticular globules on the spore wall. Fig. 14, from the same cone, upper megaspore zone. (Nos. 32d and 23b resp., Sedgwick Museum.) Fig. 15, from the type specimen of '*Lepidostrobus hibbertianus*' Binney (No. 473d, Sedgwick Museum). Fig. 16, from cone No. K1 (Slide K1a, Geology Dept., Bedford College).

TEXT-FIGS. 17A-E. Microspores of *L. russelianus*, $\times 1,000$. A-C, from the type specimen (Nos. 32y and 32z, Sedgwick Museum). D-E, from cone No. K1 (Slides K1x, K1y, Geology Dept., Bedford College).

missing from the exposed surface of the cone; but they are visible in profile at the cone margin (both in the actual specimen and in Binney's figure of it) in the upper part of the cone, especially at the left-hand side. Some of them can be seen in Binney's figure to be at least 8 mm. long: that is, very much longer than the supposed 'scales' shown in surface view in his Fig. 2. This cone does not, then, differ in this or in any other significant respect from the other specimens of *L. russelianus*.



TEXT-FIG. 18. Histogram of megaspore size in *Lepidostrobus russelianus*, based on the contents of 5 cones.

(d) *The spores.* One of the most interesting features of *Lepidostrobus russelianus* is the occurrence in the type specimen of alternating zones of megaspores and microspores. Each zone is quite clearly delimited; the lower megaspore zone extends to the base, so far as this is seen; above this, there is a zone of at least 2 cm. of microsporophylls; then nearly 1 cm. of megaspores, above which the remainder of the cone is of microsporophylls up to the truncated end of the cone. The microspores from both parts of the cone are essentially the same in all structural features, but rather surprisingly, those from the upper zone have a mean size range slightly greater than that of the spores from the lower microspore zone (histogram, Text-fig. 19 E). The megaspores from the upper megaspore zone are appreciably smaller than those from the lower zone; the mean diameter of 13 megaspores from the upper zone being 620μ (range $510\text{--}700\mu$), while 15 from the lower zone have a mean diameter of 740μ (range $650\text{--}840\mu$). Typical spores from the two zones are shown in Text-figs. 13 and 14.

The megaspores from *Lepidostrobus russelianus* (Text-figs. 13–16) agree well with the data given by Dijkstra (1946) and Arnold (1950) for *Triletes rugosus* (Loose) Schopf. They conform most closely with the first of the three groups delimited by Dijkstra (1946) within the species. The globules sometimes present adhering to the walls of the spores of *L. russelianus* are closely similar to those described for *L. dubius* (Text-fig. 13A).

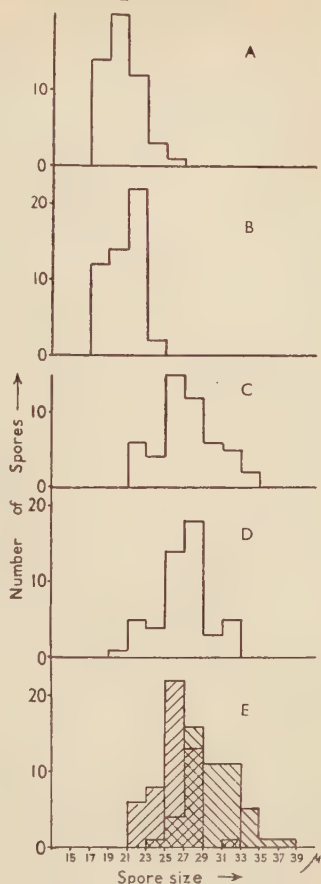
All the microspores are flattened, unlike those in the two specimens of *L. dubius* mentioned above, and it is not easy to distinguish the focal planes of the proximal and distal faces of the spore. But the papillate decoration

appears to extend only over the distal spore surface. Typical spores from two cones are shown in Text-fig. 17: they do not appear to belong to any previously described species of *Lycospora*. They are closely similar to those of *L. dubius*, and differ from them only in being rather larger. (See histograms, Text-fig. 19.)

(e) *Comparison with other species.* The only previously described cone that is at all similar to *L. russelianus* is the specimen described by Renault (1896) as the cone of *Lepidodendron esnostense*. This cone, which is known only as a petrification, is very much larger (diameter *c.* 7 cm.) than any specimens of *Lepidostrobus russelianus*. The spore from this cone petrification, figured by Renault (p. 182, Fig. 35), certainly appears to be of the *Lagenicula* type, but the spore wall is very much thicker than that of *Triletes rugosus*. Dijkstra (1946) suggests that the spore of *Lepidodendron esnostense* may be close to *Triletes nudus* Schopf rather than *T. rugosus*. (The only difference between these two species of *Triletes* is, in fact, the thicker spore wall of *T. nudus*). I therefore regard the cone of *Lepidodendron esnostense* as distinct from *Lepidostrobus russelianus*.

The cone figured by Vernon (1912) as ‘? *Lepidostrobus russelianus*’ was also assigned by Arber (1922) to this species, as emended by him. However, Arber’s interpretation of this species clearly included both *Lepidostrobus dubius* and *L. russelianus* as emended above. Without examining Vernon’s cone, it is impossible to assign it to one or other of these two species.

Lepidostrobus russelianus, *L. allantonensis*, and *L. dubius* can only be distinguished on the basis of their spore content. Although the size range and structure of the microspores of two of the three species differ slightly, the most convenient character for separating the cones is the type of megaspore contained in them. It is not necessary to macerate megaspores from the cones in order to separate the first two from the latter species, for examination of the basal part of the cone with a lens (or better, a binocular microscope) will show whether or not the spores are spinose. As seen in a



TEXT-FIG. 19. Histograms of microspore size in *Lepidostrobus dubius* and *L. russelianus* (each population is of 50 spores). A and B, microspores from the two hermaphrodite *L. dubius* cones mentioned in the text. C-E, *L. russelianus*. C is a population from cone No. 46, Sedgwick Museum. D is from cone No. K1, Geology Dept., Bedford College. E consists of superimposed histograms of the microspores from two levels in the type specimen of *L. russelianus*. (The area with diagonal shading to the left, lower microspore zone; area diagonally shaded to the right, upper microspore zone.)

cone compression, the spines appear as small apiculae or projections on the surface of the spores of *L. dubius* and *L. allantonensis*, whereas in *L. russelianus* the megaspores appear either smooth or slightly rugose. But transparent preparations are necessary to separate the two former species.

Since the distinguishing characters of all three of these *Lepidostrobus* species depend on the megaspores, a problem arises in connexion with the determination of empty cones. Arber (1922) recognizes only two species of cone similar to the three described. One, containing megaspores, for which he used the name *L. russelianus* in a very broad sense (in which he 'lumped' Binney's *L. russelianus*, and *L. dubius*), and the other, *L. variabilis* L. & H., lacking evidence of megaspores (and so presumed by that author to contain microspores). While I reject Arber's use of *L. russelianus* since it ignores megaspore structure, I believe that *L. variabilis* must be retained as a designation for cones which, lacking evidence of their spore contents (which may have comprised megaspores, microspores, or both), cannot be assigned to a more precise group. Empty cones, which could have been assigned to *L. dubius*, *L. russelianus*, or *L. allantonensis* if their megaspores had still been present, must be placed in this species, *L. variabilis* as emended by Arber (1922).

(f) *The age of 'Lepidostrobus hibbertianus' Binney, 1871.* Amongst the specimens described and figured by Binney (1871) is the cone that he made the type of the species *L. hibbertianus*, and which I have assigned to *L. russelianus*. (A specimen of *Triletes rugosus* from this cone is shown in Fig. 15.) I know of no records of this species apart from the type specimen. Binney gives its horizon as being 'the Burdiehouse Limestone, from near Edinburgh'. He states that the specimen 'came from the collection of the late Dr. Hibbert of Edinburgh, and was purchased by me at the sale of his museum' (p. 55). Unlike the other (Airdrie Blackband) specimens described by Binney in the same monograph, it did not come to him directly from Mr. Russel, the collector at Airdrie. But this cone, although reputed to be of Lower Carboniferous age, is indistinguishable in all details of structure and megaspore contents from the Upper Carboniferous specimens of *L. russelianus* from Airdrie. Also, in spite of its reputed source being the Burdiehouse Limestone, the matrix is completely non-calcareous, and has a remarkable similarity in appearance and streak to the Blackband Ironstone of Airdrie. Further, on the back of this specimen there is a number of Lamellibranchs closely similar to those on some of the Blackband material. Dr. Eager of Manchester, who has examined the specimen, comments (written communication, Nov. 1951): 'I cannot believe that the label (which states that the specimen is from the Burdiehouse Limestone) is correct. Indeed, they (the mussels on the back of the specimen) are what you might expect from the Airdrie Blackband.' On the basis of both this palaeontological evidence and that of the character of the matrix, I believe that this specimen is not in fact from the Burdiehouse Limestone, and is probably from the Blackband Ironstone of Airdrie, Scotland. I have therefore placed '*Lepidostrobus hibbertianus*' Binney in the Upper Carboniferous species, *L. russelianus* emend.

(g) *The association of Lepidostrobus russelianus with Lepidodendron acutum* Presl. In addition to the cones cited above, I have collected 50 cones agreeing superficially with *Lepidostrobus russelianus* from the Forest of Dean Coalfield (Northern United Colliery, roof of the Coleford High Delf seam, ? *phillipsi* zone, Westphalian C). Ten of these cones have yielded a number of *Triletes rugosus* spores, so confirming the attribution of the cones to *L. russelianus*. Four of the remaining cones yielded abundant microspores agreeing closely with those described from *L. russelianus* (above). Forty of the fifty cones have on the same pieces of shale, fragments of *Lepidodendron actum* Presl. sensu Nemejc, 1947. This species of *Lepidodendron*, as revised by Nemejc (1947), includes *Lepidodendron lanceolatum* Lesq., sensu Arber, 1922. Dr. Crookall has very kindly examined a specimen of the *Lepidodendron* associated with these *Lepidostrobus russelianus* from the Forest of Dean, and agrees with its determination as *Lepidodendron lanceolatum* sensu Arber (1922) (i.e. *L. acutum* sensu Nemejc, 1947). Leafy shoots associated with the fragments of bark have leaves up to 3 mm. broad by 1.3 mm. long. This supports the attribution of this species to *L. acutum*, as Nemejc (1947, p. 67) regards the broad leaves as an important character of *Lepidodendron acutum*, distinguishing it from the narrower leaved *L. simile* and *L. ophiurus*. Nemejc records having found cones up to 15 mm. diameter in connexion with *L. acutum*. These could clearly be of the *Lepidostrobus russelianus* type, but he gives no account of their spore contents.

The specimen of *Lepidostrobus* from the Forest of Wyre (Kidston Collection, No. 5547), mentioned above, also has a piece of *Lepidodendron* on the shale matrix, determined by Kidston as *L. lanceolatum* (i.e. *L. acutum*, sensu Nemejc). The cone from Michigan, described and figured by Arnold (1949), which I have assigned to *Lepidostrobus russelianus* was stated by that author to 'evidently belong to *Lepidodendron lanceolatum*' (*L. acutum*, sensu Nemejc). Whether this is based on association or the identity of the short piece of leafy shoot attached to the cone is not stated.

There are thus records of association of *Lepidostrobus russelianus* and *Lepidodendron acutum* Presl. sensu Nemejc from three localities. The association from the Forest of Dean locality is particularly striking. On the basis of this evidence it seems extremely probable that *Lepidodendron acutum* Presl. sensu Nemejc bore cones agreeing with *Lepidostrobus russelianus*.

LEPIDOSTROBUS OLYRI ZEILLER

1886 *Lepidostrobus olyri* Zeiller (Pl. LXXVII, Fig. 1.)

1889 *Bothrodendron minutifolium* Kidston (Pl. IV, Fig. 6.)

1904 *Bothrostrobus olyri* Zalessky (Pl. VI, figs. 4 and 11, part and counter-part)

1910 *Bothrostrobus olyri* Renier (Pl. 16, b).

Emended diagnosis

Cone cylindrical, 6.0–12.0 mm. wide, and up to 10 cm. long, tapering to a rather obtuse point at the apex. Cone axis 1.5–2.2 mm. diameter, bearing

sporophylls in whorls, or in a low-angle spiral: distance between the whorls or turns of the spiral, 1.0–1.5 mm. Each whorl or turn composed of up to 8 sporophylls. Sporangia about 1 mm. high, 2.5 to 4.0 mm. long. Sporophyll laminae closely appressed to the cone; triangular, with an acute point at the apex: 2.0–3.0 mm. broad at the base, and up to 5 mm. long.

Megaspores belonging to *Triletes rugosus* Loose (Schopf). Shape, spheroidal, with a well-developed apical prominence. Mean maximum diameter of the flattened spore, 833 μ . Apical prominence formed by expansions of the contact faces; typically 360 μ high (from apex to arcuate ridges) with a range from 300 to 430 μ ; 330 μ wide, range 270 μ to 420 μ . Arcuate ridges well developed. Walls typically 10 μ thick, range 9 μ to 16 μ . Flaps forming the apical prominence rather thicker. Spore wall finely granular, and some spores pitted with hollows in the outer surface, with occasional globules of cuticular material adhering to it. Short outgrowths present on some spores, especially in the equatorial region, up to 68 μ long, 32 μ diameter, with rounded, sometimes swollen apices.

The flattened microspores, circular in outline, have a mean maximum diameter of the flattened spore of 26 μ (range 19 to 34 μ , for 100 spores measured). Triradiate ridges present, but not prominent, reaching almost to the spore margin. Proximal face of the spore flattened (in the uncrushed spore) but the equatorial thickening very slight. Entire surface decorated with minute blunt papillae.

Type specimen. The specimen figured by Zeiller, 1886 (Pl. LXXVII, Fig. 1).

Range. England: Lancashire and Yorkshire coalfields, within the *similis-pulchra* zone only (Westphalian C and B).

France: Valenciennes Coal Basin.

Further specimens of this species. Megaspores were obtained from the following specimens: V 30570, British Museum, Natural History, London. From the Barnsley Bed, Yorkshire, England. (Pl. VIII, Figs. 4 and 5). Nos. 2716, 2717, 4224, 3461, 1738, Kidston Collection, Geological Survey Museum, London. All from the Barnsley Bed, Barnsley, Yorkshire. L12415. Manchester Museum. From 'the Roof of the Four Foot Mine, ? Tonge, near Bolton?' (*similis-pulchra* zone), Lancashire, England. Of the nine cone fragments on this specimen, four yielded megaspores, and one microspores. Microspores were obtained from the following specimens: WH/2773, British Museum, Natural History, London. From the Barnsley Bed, Yorkshire, England. Nos. 3466 and 2901, Kidston Collection, Geological Survey Museum, London. From the Barnsley Bed, Yorkshire, England.

Discussion

(a) *The spores:* Since the spore contents of the type specimen are unknown, the cones described in this paper have been assigned to *Lepidostrobus olryi* on the basis of the characters of the cones themselves. Although megaspores have been obtained from ten cones, and microspores from four, there is no

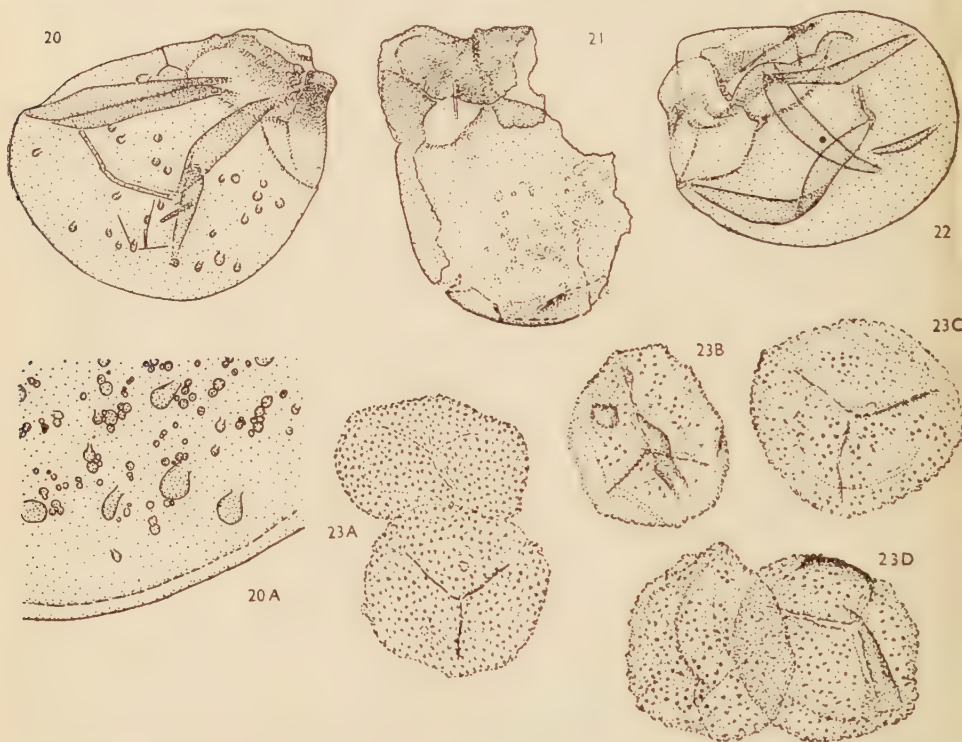
clear evidence as to whether the cones of this species were hermaphrodite, unisexual, or that cones of both types were borne by the same species of parent tree. Most of the specimens of cones are incomplete at one or other end, and so throw no light on the problem. The only intact cone which contained spores (No. V2773, British Museum, Natural History) gave microspores alone. On the basis of the evidence at present available, I consider that the characters of the cones alone are sufficient for them to be placed in one species. Pl. VIII, Fig. 5 shows a typical specimen, showing the arrangement of the sporangia, and the short sporophyll laminas. Pl. VIII, Fig. 4 is of the same cone 'shadowed' with ammonium chloride, to show the broad axis, and the prominently whorled arrangement of the sporophylls. Fig. 21 shows a megaspore from this cone.

The megaspores of *L. olryi* have a size distribution and mean very similar to those from *L. russelianus*, but show a greater range in the decoration of the spore wall (Text-figs. 20–22). Although most of the spores from *L. olryi* agree in all respects with *Triletes rugosus* (sensu Dijkstra), others show a development of appendages which has never been previously described for that species. Nevertheless, these spores, if found isolated, would be rightly assigned to *T. rugosus*. Arnold (1950, p. 82) describes the occurrence on spores which he assigns to '*Lagenicula rugosa*' (*Triletes rugosus* of Dijkstra) 'scattered, minute papillae, which are $5\ \mu$ broad, and $9\text{--}10\ \mu$ long, and have globular tips' and adds '... but their sporadic occurrence does not seem to constitute a basis for specific separation'. Although papillae of this size were observed on some of the spores of *L. russelianus*, the papillae on some of the *L. olryi* spores are considerably larger (up to $68\ \mu$ long, and $32\ \mu$ diameter) with globular heads (cf. Text-fig. 20A). Since this decoration is present on some spores and absent from others within the population of a single cone, they cannot be used as a basis for specific separation.

The microspores from the four specimens of this species which have yielded them form a uniform population (cf. Text-figs. 23A–D). Only three cones yielded sufficient spores for histograms to be plotted, and these show a very similar type of size range as the microspores of *L. russelianus*. They differ slightly from the spores of that species in having only a very slight thickening of the equatorial region, which is apparent only in spores squashed obliquely to the equatorial plane (cf. Text-fig. 23D). These spores, if found isolated, might not be placed in *Lycospora* (as would the microspores of the other two species of *Lepidostrobus* described in this paper), owing to the equatorial flange being so weakly developed, but might possibly be placed in *Planisporites* Knox or *Verrucosporites* Knox (1950).

(b) *Comparison with other species.* *Lepidostrobus olryi* may be distinguished from *L. russelianus* in being considerably smaller (typically 8 mm. wide, against 1.6 mm. for the latter species), and in having a relatively thick axis. The megaspores of *L. olryi* have in some cases the rather large swollen papillae (absent from those of *L. russelianus*) and the microspores lack a prominent marginal rim.

(c) *The affinity of Lepidostrobus olryi*. One of the most interesting aspects of *L. olryi* is the affinity of its parent plant. Zeiller evidently believed it to have been borne by a species of *Lepidodendron*. But Kidston (1889, Pl. IV, Fig. 6), who first published a figure showing this species in connexion with the fine leafy shoots with which it is often associated (cf. Pl. , Fig. 5), believed these



TEXT-FIGS. 20-22. Megaspores of *Lepidostrobus olryi* Zeiller, $\times 50$. Fig. 20, slide No. 2717a, Kidston Collection. Fig. 20A, part of the same spore, $\times 150$, to show the swollen outgrowths, and the cuticular globules. Fig. 21, from the cone in Pl. VIII, Figs. 4 and 5, slide No. V./30570A, Brit. Mus. Nat. Hist. Fig. 22, from a specimen in the Manchester Museum, slide No. L12415/Id.

TEXT-FIGS. 23A-D. Microspores of *Lepidostrobus olryi*, $\times 1,000$. A and B from slide No. 2901a, Kidston Collection. C, D, Brit. Mus., Nat. Hist., No. W.H./2773A.

shoots to be those of *Bothrodendron minutifolium* and so assigned the cone to that species. Zaleski (1904) evidently doubted the certainty of this interspecific correlation of cone and parent tree, and suggested that it would be less of a pre-judgement of the relationship to refer to the cone as '*Bothrostrobus olryi*'. Subsequently, Hemmingway (footnote in Crookall, 1929, p. 26), who collected Kidston's figured specimen, suggested that Kidston had attributed this cone to *Bothrodendron minutifolium* in error, and that in fact it belonged to *Lepidodendron lycopodioides*.

Relatively few figures have been published showing the leafy shoots of

Bothrodendron minutifolium in organic connexion with determinable branches of that species. I know of only two: that of Zeiller (1886, Pl. LXXIV, Fig. 4) and Lindsey (1915). I have compared Kidston's cone and leafy shoot (figured, Kidston, 1889) with Lindsey's figured material of undoubted *Bothrodendron minutifolium* (now in the Manchester Museum), and there is certainly no close agreement between the leafy shoots on which *L. olryi* was borne and those attached to stems showing *Bothrodendron minutifolium* leaf scars. The leaves of the *Bothrodendron* are considerably wider, and are more nearly ovate in outline (as shown in Zeiller's Fig. 4, Pl. LXXIV, 1888) than those on the shoots bearing *L. olryi*. Also the leaves on the shoots of the *Bothrodendron* are more squarrose in their arrangement and the leaf-covered shoots are broader. Comparison with Zeiller's figures (1886) suggests that the leafy shoot attached to *Lepidostrobus olryi* (cf. Kidston, 1889) agrees more closely with *Bothrodendron punctatum* or *Lycopodites carbonaceus* than *B. minutifolium*. It also agrees equally well with the figure published by Kidston (1884, Pl. V, Fig. 5) of a specimen attributed to *Lepidodendron elegans*. But the label on the specimen (now in the Geological Survey Museum) on which this figure was based was later corrected by Kidston to *L. lanceolatum*. There is thus considerable difficulty in determining these fine leafy lycopod shoots, which, since the cuticles are not preserved, have very few constant diagnostic characters by which they may be identified. This is emphasized by Nemejc (1947, p. 72), who ascribes to '*Lepidodendron* (?*Bothrodendron*) *selaginoides* Stbg.' several of the specimens attributed by Kidston to *Bothrodendron minutifolium*.

It therefore seems desirable to retain the more generalized term '*Lepidostrobus olryi*' Zeiller rather than to ascribe this cone to *Bothrodendron*, much less a particular species of the genus. The only other Carboniferous cone ascribed fairly certainly to *Bothrodendron*, and from which spores have been obtained, is that of *B. mundum* (Watson, 1908). In this cone the spores appear rather similar to *Triletes praetextus* Zerdnt, and to belong to the Zonales group of *Triletes*, whereas the spores of *L. olryi* belong to the *Lagenicula* group. If in fact *Lepidostrobus olryi* is the cone of a *Bothrodendron*, then this genus must have produced megaspores of at least two very different kinds. But the close agreement of the megaspores of *Lepidostrobus olryi* and those of *L. russeianus* (for which there is evidence of affinity with *Lepidodendron*) strongly suggests that *Lepidostrobus olryi* is a *Lepidodendron* cone.

DIAGNOSTIC CHARACTERS IN LYCOPOD CONES

Kidston (1897) stated that 'the shedding of the bracts at maturity seems to be a characteristic of *Sigillaria* cones and one of the distinguishing points between them and *Lepidostrobus*'. Several specimens of *Lepidostrobus dubius* are known in which the lower sporophylls have been shed (cf. the type specimen, figured by Binney, 1871, Pl. IX, fig. 3). I do not know whether the sporophylls became detached before or after the cones fell from the tree. Since *Lepidostrobus dubius* cones are known in connexion with *Lepidodendron*,

it seems that the shedding of the sporophylls cannot be used on its own as a reliable character for separating cones of *Sigillaria* and *Lepidodendron*.

Bochenski (1936, p. 230) has suggested that a whorled arrangement of the sporophylls may also be a diagnostic character of *Sigillaria* cones. Schopf (1941) has challenged this on the basis of several reports of *Lepidostrobus* specimens with whorled appendages. The specimen of *Lepidostrobus dubius* shown on Pl. VIII, Fig. 3, gives clear evidence of a whorled arrangement of the sporophylls, but it is in all other characters typical of the species. This is additional support for rejecting the use of the character of whorled sporophylls on its own as a reliable diagnostic for distinguishing *Lepidodendron* and *Sigillaria* cones.

SPORE VARIATION AND CONTAMINATION

When spores are obtained from a compression of a fructification, there is always a possibility that they may not represent its original contents but are extraneous in origin. Work by Leclercq (1938) and more especially Moore (1946) has shown considerable variation in the type of microspores that may be obtained by maceration of any one such fructification. Moore attempted to explain this variation as representing developmental stages of the microspores, and was subsequently criticized by Knox (1949) and Crookall (1949), who suggest that this variation may be due to contamination. I agree with the latter authors. Where there is a risk that extraneous spores may be present in a fructification, then the onus rests with the worker concerned to demonstrate that the spores obtained from it are its original contents since this relationship cannot be assumed automatically.

In the case of the material described in this paper, there is convincing evidence that the microspores ascribed to the three cone species are not extraneous in origin. In all cases where microspores were obtained in any quantity, there was a clear-cut demarcation between a zone containing clearly visible megaspores (normally in the lower part of the cone) and a relatively uniform zone which contained microspores (normally in the upper part of the cone, but see p. 280). Maceration of a minute fragment from the microspore zone yielded only large numbers of spores, all very similar, and a few fragments of cellular membrane. The microspores from any one cone were all the same in their general structure, wall thickness, and decoration. The sizes of microspores from a single cone, plotted as a histogram, always showed a variation suggesting a single population (cf. Text-figs. 19A-E). Those from different parts of a single cone showed a similar agreement (see Text-fig. 19E). Even where microspores from the apex of a cone were found to be slightly larger on the average than those from a lower point on the cone, the spore sizes all fell within 30 per cent. of the mean. In all other respects the microspores from the apex agreed with those from the lower part of the cone, there being no suggestion of thinner walls or weaker development of the decoration, as might have been expected had they been immature.

The most striking evidence in the present material that the microspores

were the true contents of the cones is the fact that cones from different localities, placed in the same species on other grounds (including agreement of the megaspores), contained the same type of microspores, with similar size-variation histograms (cf. Figs. 19A, B).

In cases where relatively few microspores are obtained from a fructification, and there is a considerable variety of forms present, then even the most common type may not represent the original contents of the cone. The presence of such spores can only be accepted as evidence of close association. For example, Leclercq (1938) figures a number of bodies alleged to show the developmental sequence of the microspores of *Sigillariostrobus sphenophylloides*, from $35\ \mu$ to $80\ \mu$ diameter. I have examined the preparations from which these figures were made, and have also made further preparations by macerating fragments from two points within the supposed microsporangial region of the cone. Only the preparations of her Pl. IV, Figs. 5, 7, and 9, are still extant, although a number of the spores of the type shown in her figs. 3 and 4 are present in the preparations. There are in fact relatively few spores in any of the preparations. The commonest type are those figured in her Figs. 3 and 4, and there are rather less than 50 of these present in all the preparations. Although there seems little doubt that these small bodies are spores, there is no evidence to indicate that they are the original contents of the cone rather than any other of the spore types present. But the spore with the thick rugose exine shown in Leclercq's Fig. 8, Pl. IV, was the only one of that kind in any of her preparations. The bodies in Figs. 7 and 9 of this plate are, I believe, not spores at all, but resinous bodies; they have no clearly defined shape, and a number of similar bodies occur in Leclercq's slides, some smaller and some larger than the two that she figures. All of these resinous bodies get progressively thinner towards their margins, and give no indication of being in the form of a flattened cellular structure such as a crushed spore (or the remainder of Leclercq's preparations, Figs. 3, 4, and 8 of Pl. IV). Dr. Knox, who has examined Leclercq's preparations, agrees that the objects in Figs. 7 and 9 of Leclercq's Pl. IV are not in fact spores but most probably resin bodies.

As well as the preparations from the two points within the supposed microsporangial region of the cone, I have made further preparations from the matrix several centimetres from the cone. Spore counts on all these preparations gave the results given on p. 290. (The slides on which these are based are Nos. 2783 *j-q* in the Kidston Collection, Geological Survey Museum, London.)

This data does not give any indication of the original spore contents of the cone, but rather indicates that both the apex of this cone and the adjacent part of the matrix evidently contain relatively small numbers of a variety of spore types; which is what might be expected in the case of contamination. None of these spores agree with the type of *Lycospora* so abundant in the apices of hermaphrodite *Lepidostrobus dubius* cones. I believe that there is no evidence that any one of these spore types, rather than any other, represents

the original population of the upper part of the cone: much less a developmental sequence of the original spores. The abrupt cessation of the megaspores in the upper part of the cone suggests either total abortion of the spores above this level or death of the cone before cutinization had proceeded far enough for the spores to survive fossilization.

	Leclercq's preparations from the cone.	Further preparations from the cone.	Preparations from the matrix.
<i>Densosporites</i> sp.	—	35	14
Thick-walled spores, aff. Leclercq's Pl. IV, Fig. 8	1	10	—
<i>Spinosporites</i> sp.	—	3	—
<i>Lycospora</i> sp.	—	6	1
<i>Calamospora</i> sp.	—	3	2
<i>Planisporites</i> sp.	—	1	—
Resin bodies	(Figs. 7, 9, of Leclercq, 1938, Pl. IV)	14	15
Small ? spores (cf. Leclercq's figs. 3 and 4, Pl. IV)	c. 50	—	—

In nearly all cases where I obtained megaspores from cones these were visible in large numbers within the cone before maceration. The risk of contamination of a cone by a considerable number of extraneous megaspores is in any case very remote owing to their relatively large size.

I have not yet found evidence in either megaspores or microspores obtained from cones of variation in structure or decoration due to immaturity. There is a certain random variation in size, even among the spores from one level in the cone (and possibly from a single sporangium); and in one case there is evidence of megaspores from near the apex being somewhat smaller than those from lower in the cone. It might be expected that immature megaspores would have thinner walls, be less resistant to maceration, and would adhere in tetrads more than the larger spores from the same cone. But the smaller megaspores mentioned above do not show these symptoms of immaturity. Duerden (1929) has shown that a comparable variation in megaspore size occurs in some living *Selaginella* species, and that this is quite independent of their relative ages. There is no indication that the smaller megaspores, either in the living *Selaginella* or these *Lepidostrobus* cones, would ultimately have reached the same size as the larger ones. I do not believe that such thick-walled bodies could have increased appreciably in size. I prefer to regard these smaller spores as starved or otherwise abnormal specimens.

SUMMARY

1. A threefold correlation is established between the 'megaspore species' *Triletes horridus* (Zerndt) S. W. & B., the 'cone species' *Lepidostrobus dubius* Binney, and the tree, *Lepidodendron simile* Kidst.

2. A new species of cone, *Lepidostrobus allantonensis*, is shown to bear megaspores agreeing with *Triletes crassiaculeatus* (Zerndt) S. W. & B.

3. *Lepidostrobus russelianus* Binney is shown to bear megaspores agreeing with *Triletes rugosus* (Loose) Schopf, and on the basis of significant association at three localities, it is suggested that *L. russelianus* was borne on *Lepidodendron acutum* Presl. (sensu Nemejc).

4. *Lepidostrobus olryi* Zeiller (attributed to *Bothrodendron minutifolium* by Kidston) is also shown to bear *Triletes rugosus* megaspores. On this and other evidence, it is suggested that *L. olryi* was not borne on a *Bothrodendron*, but that it was most probably a *Lepidodendron* cone.

5. Although two 'megaspore species' are shown, so far as the present material is concerned, to have been borne by two corresponding 'cone species', one of the three 'megaspore species' investigated (*Triletes rugosus*) is shown to have been borne by two quite distinct 'cone species'.

6. The microspores from three species of *Lepidostrobus* are described and figured: all belong to the isolated 'microspore genus' *Lycospora* S. W. & B. Histograms of the microspore populations of the cones show a relatively limited size variation within a species. This is in marked contrast to records of some authors who claim to have demonstrated 'developmental series' in fossil spores, involving considerable changes in spore size and structure.

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EXPLANATION OF PLATE

Illustrating W. G. Chaloner's article 'On the Megaspores of Four Species of *Lepidostrobus*'

FIG. 1. Shoots of *Lepidodendron simile*, with attached cones (*Lepidostrobus dubius* Binney). The cone in the upper central part of the figure, attached to the shoot, yielded the *Triletes horridus* spore shown in Text-fig. 6. From the roof of the Upper Foot Mine, Hulton Collieries, Lancashire (*lenisulcata* zone). No. L10878, Manchester Museum. ($\times \frac{1}{2}$.)

FIG. 2. *Lepidostrobus allantonensis* sp. nov., type specimen. The whorled sporophyll scars can be seen on the upper part of the cone axis. The specimen of *Triletes crassiaculeatus* in Text-fig. 12 was obtained from this cone. From the Whiteadder Water, 1 mile east of Allanton Bridge, Berwick, Scotland. No. 2972, Kidston Collection, Geological Survey Museum, London.

FIG. 3. A plasticine cast of the upper part of a specimen of *Lepidostrobus dubius* coated with ammonium chloride, and photographed with oblique illumination. The ends of the sporangia show as rounded projections, arranged in alternating whorls, and producing a corresponding vertical seriation. From the Coal Measures, horizon unknown. The original specimen (from which *Triletes horridus* was obtained) is No. Z74 in the Collection of the Botany Dept., Royal Holloway College (University of London), Englefield Green, Surrey.

FIGS. 4 and 5. *Lepidostrobus olryi* Zeiller. Fig. 4, photographed with the specimen coated with ammonium chloride, shows the whorled arrangement of the sporophylls, and the relatively broad axis. Fig. 5: the same specimen, photographed under paraffin oil, shows the shape of the sporophylls and sporophyll laminas, and some of the fine leafy shoots associated with the specimen. From the shale over the Barnsley Bed, Royston, Yorkshire. No. 30570 in the British Museum, Natural History, London.



W. G. CHALONER

The Structure and Development of the Cell-Wall in the Valoniaceae as Revealed by the Electron Microscope

BY

F. C. STEWARD

AND

K. MÜHLETHALER¹

(Dept. of Botany, Cornell Univ., Ithaca, N.Y., and National Institute of Health,
Bethesda, Md.)

With Plates IX to XVII

ABSTRACT

The cell walls of *Valonia ventricosa*, *V. macrophysa*, *V. ocellata*, and *Dictyosphaeria favulosa* have been investigated with the electron microscope. The pattern of wall structure and development appears to be similar in all.

The first wall consists of a tangle of cellulose fibrils embedded in amorphous material, possibly pectin. Later, cellulose is laid down in successive lamellae in which the fibril direction shifts abruptly from layer to layer apparently through 120°, so that the direction is repeated on every fourth layer. During cell-extension, tearing of the lamellae occurs. The implication of the observed facts is discussed.

SINCE the statement of the micellar concept by Nägeli in 1858 the sub-microscopic structure of the plant cell-wall has been of the greatest interest to students of the structure of matter and of plant anatomy. Cellulose, wherever deposited in the plant cell-wall, exhibits regularity of structure and a sub-microscopic molecular architecture, which is revealed by the use of the polarizing microscope, by the techniques of X-ray diffraction, and by the electron microscope. This fact has brought new tools to the aid of morphology and has bridged the erstwhile gap between it and physical science. The attention which has been devoted to the cell walls of algae, and notably of the Valoniaceae, has played an important role in these developments ever since Correns (1893) made his well-known examination of the intimate structure of the algal 'membrane' or wall. Since that time many investigations, more or less comprehensive, have made use of the wall of *Valonia* as the test object.

Preston (1931) and later Farr (1949), using the species *V. ventricosa*, followed Correns (1893) in the application of the compound and polarizing microscopes to the wall of *Valonia*.

¹ Present address: Laboratorium für Elektronmikroskopie, Universitätsstraße 2, Zürich, Switzerland.

The technique of X-ray diffraction, used to propose a three-dimensional molecular model of fibre cellulose by Sponsler and Dore (1926), was first applied by Sponsler (1931) to the wall of *Valonia*. Sponsler established the fact that this wall exhibited regularity of structure comparable to that found in the fibres of higher plants with cellulose chains lying regularly and roughly parallel to the surface of the wall. Since then this organism has been investigated by X-ray diffraction methods by Astbury, Marwick, and Bernal (1932); Preston and Astbury (1937); Sisson (1941), and the results so obtained have prompted speculation on the processes involved in wall formation. An admirable account of the history of the X-ray examination of cellulose fibres is given by Sisson (1943); also see Bonner (1951).

With the development of electron microscopy the study of the structure of cellulose, regarded as a chemical substance, and of the organization of the plant cell-wall took a new turn. Preston, Nicolai, Reed, and Millard (1948) published a first account of the wall of *Valonia* (*V. ventricosa*) by the aid of the electron microscope, to be followed by other studies by Wilson (1951) and by Preston and Kuyper (1951)—the last two becoming available after the work now to be described was already far advanced.

Most of the investigations on *Valonia* wall have been made upon dried (i.e. herbarium) specimens or pickled material. The first work of Astbury and Preston *et al.* (1932, 1937) was done upon what appears to have been a single *V. ventricosa* vesicle which had been preserved as an herbarium specimen. Later Preston (1951) and Wilson (1951) made use of some *Valonia* collected by one of us (F. C. S.) in the Dry Tortugas, Fla., and preserved in formalin sea-water. At least in one case (that of Wilson) particular advantage was taken of the partial maceration which the vesicles had undergone during the long period (over 10 years) of storage in a preservative. Even where investigators (Farr, 1949) have had access to living material, apparently shipped to the laboratory from Bermuda, the descriptions of it hardly show a degree of familiarity with the organism and the way it grows which seems necessary for an understanding of the development of the wall.

In a previous investigation (Steward, 1939), two species of *Valonia* were maintained in laboratory culture for long periods and vesicles were grown from minute, naked protoplasmic masses (aplanospores). This led to the view that a developmental study of the wall would be necessary to determine the full details of its molecular structure (*loc. cit.*, p. 90).

The purpose of the present collaboration was to bring to bear upon this problem the experience of a laboratory well versed in electron microscopy, the experience of an investigator (K. M.) already familiar with plant cell-walls as revealed by the use of the electron microscope, and the experience gained with these organisms both in their habitat (Steward and Martin, 1937) and under culture conditions (Steward, 1939). Methods had been worked out (Steward, 1939) by which two species could be, not merely kept alive, but cultured from spores, thus permitting the wall to be investigated as it develops.

Of the known species and genera of the Valoniaceae, four were used in this

investigation: these were *V. ventricosa* J. Agardh., *V. macrophysa* Kuetzing, *V. ocellata* Howe, and *Dictyosphaeria favulosa* (C. Agardh) Decaisne. All of these plants occur in the Dry Tortugas, Fla. (Taylor, 1928), whence they were obtained. The most important feature of the work was made possible by the propensity of these organisms to form aplanospores from which mature vesicles can be grown under culture conditions. It was thus possible to follow the developmental stages which intervene between the first formation of cellulose upon a new, naked, protoplasmic surface and the subsequent development of the lamellated cellulose wall typical of the mature vesicle.

With *V. ventricosa* at the Dry Tortugas, aplanospore formation in large vesicles is a frequent occurrence in its natural habitat, and especially so in the larger vesicles, which may reach a volume of over 30 c.c. Apparently aplanospore formation is the natural method of propagation of these plants (Steward, 1939, p. 95) in this habitat. There is no record of the formation by the Tortugas material of the zoospores described by Kuckuck (1907) for other species and localities. Be that as it may, the formation of aplanospores by artificial stimuli as described by Kopac (1933, 1937) and Steward (1939) provides a more satisfactory means of procuring sporelings than reliance upon their occasional occurrence and collection. In *V. ocellata*, on the contrary, the natural occurrence of aplanospores, within the old vesicle, and in various stages of development towards the 'morulloid mass' which they eventually form by mutual compression, is sufficiently common to be a satisfactory source of sporeling material. For some unexplained reason the formation of aplanospores in *V. macrophysa* by external stimulation is much more difficult, though it occasionally occurs by natural means.

The non-motile aplanospores, which are formed in very large number by *V. ventricosa*, and quite readily by *V. ocellata*, present at their formation a naked, protoplasmic surface. At first the outer membrane has the properties of a liquid and will freely ingest relatively large liquid drops of appropriate surface tension (Kopac, 1937). Quickly thereafter (in a matter of hours) the character of the surface changes, it loses its unequivocal liquid nature and behaves as a solid pellicle. The green sporelings may grow and increase their volume considerably, even while they are within the old vesicle, and when isolated, as in a dish of sea-water, they will survive for long periods. For continued growth the sporelings of *V. ventricosa* must be attached to a substrate and kept in diffuse light; the substratum which has proved successful consists of small marble blocks in sea-water (Steward, 1939).

INVESTIGATIONS ON *VALONIA VENTRICOSA* J. AGARDH

This organism forms a single, large (up to 30 c.c. or more) vesicle, completely unbranched except for the stout rhizoidal holdfasts and the more numerous thin-walled filaments which develop at the base from the crowded 'lentiform' cells of Børgesen (1913) or the 'Uhrglaszellen' of Oltmanns (1922) and Kuckuck (1907). Details are given by Steward (1939, p. 93).

The vesicle can be regarded as enclosed within a single, almost spherical, envelope which must develop progressively from the first membrane formed around the minute aplanospores.

Material of *V. ventricosa* was collected during August 1951 at various points around Loggerhead Key, Dry Tortugas, Fla. The large vesicles are commonly somewhat encrusted with calcareous material and bear epiphytes. These were removed and the cleaned vesicles kept alive in sea-water until required for the electron-microscopic study of the mature wall.

On suitable vesicles aplanospore formation was stimulated, either deliberately by the method of minute punctures as practised by Kopac (1937), or as a result of similar minute punctures incidental to the removal of calcareous and other materials. The first definite appearance of the aplanospores could be readily observed with a lens or the microscope and the time that had elapsed from the original stimulus estimated. In this way a population of aplanospores of known age was obtained. The aplanospores were then allowed to develop undisturbed for recorded time-intervals: up to a few days in dishes of sea-water, or for several weeks after sowing them on marble blocks in sea-water. Aplanospores thus obtained were examined at their first appearance, at 15 hours after the original stimulus for their formation, and at intervals until they were 12 weeks old.

The cell walls were prepared in a form suitable for electron microscopy as follows:

The oft-noted and pronounced lamellation of the wall of the mature vesicle makes it easy to prepare specimens by simply 'tearing' or 'stripping' the wall of vesicles with a needle under the microscope. This can be done very readily on vesicles which have been in boiling 6 per cent. HCl for 30 minutes and are then washed with water. The minute wall-fragments were mounted on a grid and shadowed in the usual way with palladium (see Williams and Wyckoff, 1946). In this manner single lamellae, i.e. sheets with strands in only one direction, are not usually obtained, but this is not a disadvantage because the electron beam can penetrate even three layers of strands (Pl. IX).

The cell wall contains an abundance of non-cellulosic, amorphous material, presumably the mucilaginous covering of aplanospores noted by Steward (1939). To obtain the clearest definition of the cellulose this material was removed by boiling in 6 per cent. HCl and, if this treatment did not suffice, it was followed by 6 per cent. NaOH (Mühlethaler, 1950a). Whilst the clarity of the completely cleared preparations is essential to the interpretation of the cellulose wall structure, the presence of the non-cellulosic, more amorphous, material in which the cellulose is embedded is equally important in the interpretation of the wall as a whole (Pl. IX (i) and Pl. X (iii)).

The electron microscope alone cannot establish the chemical nature of the amorphous material in the wall. There is a presumption that it is pectin-like, strengthened by the results of the chemical tests of Mirande (1913) and of Van Itersen (1936b). Hence the amorphous material is regarded as pectin

since cellulose, wherever present, is always in the form of a well-defined fibril (Mühlethaler, 1950b).

The sporelings were especially suitable for electron microscopy as they were free from epiphytic calcareous incrustations. They were fixed in 2 per cent. formalin in sea-water for 1 hour, washed in tap-water several times, and then treated as follows:

(i) The wall was examined by transmission, i.e. as it is shown in surface view, with and without clearing it as described above.

(ii) The spore was examined in section by proceeding as follows: The cells were dehydrated in pyridine and embedded in a mixture of methyl and butyl methacrylate. The sectioning was done after the method of Newman, Borysko, and Swerdlow (1949), the methacrylate dissolved, and the sections shadowed with palladium. These sections, cut across the wall, show the arrangement of the cellulose very clearly. In these preparations individual cellulose fibrils can be seen for a distance that depends on the angle they make to the plane of the section and on the thickness of the section. Because the sections were so thin, this distance is usually short for the fibrils of a given lamella. A limited number of individual wall lamellae can, however, be studied over a much wider field by the preparation of the wall for transmission.

The combination of the teased preparations examined by transmission with and without clearing, and of the cross-sections of the wall of sporelings gives a more complete picture of the submicroscopic structure of the wall of *Valonia ventricosa* at various stages of development than any available hitherto. Selected examples from a very large number of preparations which were examined and photographed will be found in Pls. IX to XIII: upon these the following interpretations are based. The new observations will be described first and their implications upon previously published work considered later.

The mature wall of V. ventricosa

All preparations examined by transmission normal to the wall surface show, in surface view, separate lamellae in which the cellulose fibrils run in three definite directions. The consistent feature is that these directions shift, from lamella to lamella, through approximately 120° . Thus the fibril direction in the fourth and seventh lamellae to be laid down tends to be parallel to that in the first, and so on. For the three such lamellae that can be seen by transmission this is plainly evident in Pl. IX (i) and (ii), and also for the many such distinct lamellae to be seen in a cross-section as in Pl. IX (iii). The latter picture also shows clearly that, when the amorphous materials are removed, the individual cellulose lamellae are distinct and separate and are not interconnected.

Thus the pattern for the continued deposition of cellulose in the vesicle of *V. ventricosa* can now be defined. The wall is laid down in successive lamellae. In each lamella the cellulose strands or fibrils have a single direction. Passing from one lamella to the next the fibril direction changes abruptly

and the shift is through an angle of approximately 120° . After three such shifts the original fibril direction is restored in the fourth lamella and the pattern repeats itself through the 40 or 50 lamellae which comprise the relatively thick wall of a mature vesicle. Therefore, one can regard a sequence of three lamellae as constituting a 'period' or 'stanza' of wall formation and, by repetition of these events, the wall is built up.

This picture of the mature wall raises, however, the following obvious problems:

- i. What is the nature of the forces that cause the shift from one fibril direction to another? Having achieved an orderly deposition of cellulose along one chain direction, what causes its cessation and resumption in another direction at 120° to the first?
- ii. What is the nature of the first-formed wall? Is this similar to the mature structure? How soon in the development of the relatively solid pellicle on a sporeling can cellulose and its definite submicroscopic structure be detected?

The investigation of wall formation in the aplanospore provides answers to the second question.

Wall formation in the aplanospore of V. ventricosa

The course of wall formation on the naked protoplasmic surface of the aplanospore can be followed in Pls. X to XIII.

Cross-sections of sporelings which were only 15 hours old show the cell to be already surrounded by a solid membrane, the structure of which indicates that wall formation can be well advanced in some vesicles, even at this early stage (Pl. XIII (iii), (v)). About 3–5 hours after the stimulus to spore formation, Kopac (1937) noted that the surface of the spore had a visibly differentiated surface layer and then the spores lost their ability to engulf liquid globules. He was, however, unable to detect cellulose micro-chemically at this early age, or even up to 30 hours (probably because the amorphous material was not removed, and also because of the very tenuous nature of the cellulose lamella).

The cross-section of a complete aplanospore at 15 hours shows it to be densely packed with chloroplasts containing starch grains (Plate XIII (v)). The entire protoplasmic contents may, however, be removed by clearing with hydrochloric acid. The wall of the whole vesicle is then so thin that it can be studied with the electron microscope (Pl. XII), though, of course, it collapses on to the screen. The first-formed wall now appears as a loose network of interlaced threads similar to the primary walls of coleoptiles (Mühlethaler, 1950b) or of plant-fibres (Mühlethaler, 1949), or to that of root meristem cells of *Zea* (Whaley, Mericle, and Heimsch, 1952). At the base of the sporeling, where it is in contact with the substratum, there is a gap in the wall (Pl. XII (i) and (iii)) which enables one to look into the vesicle. Examining the inner surface of the sporeling in this way one can see local areas of deposi-

tion of a thicker and denser wall (Pl. XII (iv)). Later the gap in the wall at the base of the vesicle is closed.

Uncleared preparations (Pl. X (i)) of 15-hour sporelings as seen by transmission show that the cellulose network is completely concealed within a dense, somewhat amorphous material (pectin) which would effectively prevent the detection of cellulose by microchemical tests as attempted by Kopac (1937) and Steward (1939, loc. cit., p. 89). Partial removal of the amorphous substances reveals the cellulose strands more or less 'cemented' together (Pl. X (iii)) and complete removal reveals a tangled weft or 'felt-like' mass of cellulose threads in which the arrangement appears to be quite random.

The tangled weft of cellulose fibrils which constitutes the first-formed wall of the *Valonia ventricosa* spore must, obviously, be subjected to great stresses as the spore grows, especially in the early stages of its development when its relative growth rate is rapid. How does the spore wall accommodate to this extension? If the original network is stretched it should become uniformly less dense: on the contrary there are denser and looser areas which have sharp boundaries (Pl. XII (iii) and (iv)), suggesting that the first-formed wall breaks and the gap is then closed by the deposition of more wall material. The occurrence of such gaps in the first-formed wall when it consists of random fibrils can be seen by transmission (Pl. X (iv)). The process is clearly analogous to the pulling apart of the fibres of felt when it is subjected to strain. The cellulose fibres of the wall are themselves almost inextensible; they can slip past each other to a limited extent, no doubt lubricated by the amorphous material, but extensive stress results in their being irregularly teased apart in the manner illustrated. These tears were not due to any mechanical or other stress to which the walls were subjected during handling, but are apparently real features of its structure. Moreover, many isolated, fringed pieces of the original 'primary' wall may still be seen adhering to the later-formed wall.

The first random arrangement of cellulose fibrils quickly gives place to a more orderly deposition. Progressive stages of this trend from random arrangement towards order may be seen by comparing Pls. X (ii), XI (i) and (ii) which show an evident trend towards parallel orientation of the fibrils as in the secondary wall of a fibre cell (Mühlethaler, 1949). This parallelism, though not yet perfect, becomes progressively more so and, as shown in Pl. XI (iii), there are already, even at 15 hours, strands laid down in two somewhat irregular directions. In this preparation, which is unusual in this, they cross at nearly 90°. In older lamellae the parallelism is so perfect and the fibrils are so close together that the strands cannot be individually distinguished beneath the mucilage (Pl. IX (i)). Even after a close parallel pattern is evident the strain to which the outer wall layer is subjected may cause them to be pulled apart (Pl. XI (iv)) in the manner already described.

The first-formed wall of the aplanospore is clearly homologous with the primary wall of vascular plants: the later lamellae with their parallel strands are clearly homologous with the successive layers of secondary wall. It is a

common concept that only the primary wall is present during cell enlargement and the secondary wall is deposited after such enlargement has ceased. This is *not* the condition in the vesicles of *V. ventricosa*, which, like the cells of the coleoptile (Mühlethaler, 1950b), must be able to expand after the wall has begun to thicken and has several lamellae of parallel cellulose strands.

The development of the wall of the spore during the first 5 days proceeds apace. In Pl. XIII (i) and (ii) the wall is shown at 15 hours in section (i) and in surface view (ii), necessarily somewhat oblique. It is evident that at this stage and in that particular sporeling only the first-formed, randomly arranged wall was present. Even at 15 hours, however, the beginning of a second layer, well separated by amorphous material, may be seen (Pl. XIII (iii) and (iv)) and the outer layer has obviously broken under the strain of enlargement. At 5 days (Pl. XIII (v), (vi), and (vii)) the wall has many lamellae (4 to 6 could be counted) and the two conspicuous layers were well separated by a broad band of amorphous substance ((vi) and (vii)).

Surprisingly, and especially so after the rapid rate of development from 0 to 5 days, the wall changed but little in sporelings which remained for 3 months on marble blocks in sea-water even though the sporelings grew very considerably. The method of expansion of this multilayered wall with its alternating lamellae of parallel strands presents a problem. The thick intervening layer (Pl. XIII (iv) and (vii)) of amorphous substance may cast a lubricant and allow for a large measure of slip between the successive lamellae which could thus move past each other. It may well be that the events which accompany aplanospore formation allow the accumulation of wall material which suffices for growth during several weeks, and for the same reason the amorphous pectin-like constituent of the wall is much more conspicuous than at any time thereafter.

The method of break and repair is an important part of the wall development. This accounts for the prevalence on the vesicles of patches of wall which readily permit it to be 'peeled': these are the remnants of wall layers which are no longer continuous, breaks in the outer layers of the wall that cannot be repaired (Pl. XIII (iv)). In the very young sporeling, when the wall consists of only one lamella with random fibrils, gaps in the wall may be filled in by the same mechanism that produces the first-formed wall. As the spore grows it is only the *inner* wall layer which is preserved intact as each new wall layer will be formed internally to the old one and stretched to its limit.

After 5 days, 4 to 6 cellulose lamellae were counted on *V. ventricosa* sporelings. It is tempting, though unproven, to regard this as an average of approximately one layer a day, by analogy with the cotton hair (Balls, 1912). In view of Anderson and Kerr's observations (1938) on the effects of constant temperature and degree of illumination on the lamellation in the cotton hair, similar experiments with *Valonia* would be interesting. It seems, however, that the events move too fast in the first 15 hours of the separate life of the aplanospore and too slowly after 5 days for there to be any very direct correla-

tion between diurnal variations of light, temperature, &c., and wall formation in *V. ventricosa*.

The outstanding features of this re-examination of the wall of *Valonia ventricosa* may, therefore, be summarized as follows:

1. The wall of the mature vesicle (comparable to the secondary walls in vascular plants) is laid down in a series of lamellae in each of which the cellulose fibrils run in a single direction. The direction of the strands changes abruptly from lamella to lamella and at each change the direction tends to shift through approximately 120° . Three such lamellae constitute a 'period' or 'stanza' of wall formation, and by repetition of this process the multi-layered wall is built up in such a manner that the principal directions tend to be repeated at every fourth lamella.

2. The wall may be studied developmentally on the surface of a naked mass of protoplasm by making use of the artificial production of aplano-spores. On these spores the first-formed wall is embedded in a slimy or amorphous mass of pectin-like material and it consists of randomly arranged, interwoven strands of cellulose, which enclose the sporeling, except for a small area at its base which is in contact with the substratum.

3. As the spore grows the new wall expands its strands lubricated by the pectin-like material, but eventually it breaks or is pulled apart. Though breaks in the first-formed random wall may be repaired, new cellulose lamellae are laid down sealing the break, and the innermost of these will always be intact. As the vesicle grows, therefore, the remnants of earlier lamellae cling to the outer surface.

4. After the first-formed wall all the subsequent lamellae have parallel strands of cellulose following the pattern described for the old vesicle.

5. These results have obvious significance for the concept of the wall as an elastic solid in the interpretation of water relations by Ursprung and Blum (1924). To stretch the cellulose lamellae without breaking them would require a force probably beyond the turgor pressure available, since these vesicles are close to osmotic equilibrium with their sea-water. (The total osmotic concentration difference between sap and sea-water is only the equivalent of about 0.05 M KCl or approximately 2 atmospheres; see data of sap composition in Steward and Martin, 1937.)

THE WALL IN OTHER MEMBERS OF THE VALONIACEAE

The structure of the wall in the other members of the Valoniaceae which have been investigated may now be described concisely, the main point being to observe the extent to which the findings for *V. ventricosa* reflect a somewhat general condition throughout the family.

The main characteristics of these organisms are:

Valonia macrophysa grows in relatively calm and sheltered water in contrast to *V. ventricosa* which is to be found where there is access to surf or well-aerated moving waters. *V. macrophysa* at Tortugas grows in the moat of Old Fort Jefferson where it was collected. For details and photographs of

this organism see Steward and Martin (1937). *V. macrophysa*, unlike *V. ventricosa*, proliferates at the apex and its wall is not so tough. The vesicles are smaller and more nearly cylindrical than in *V. ventricosa*.

Valonia ocellata is a relatively inconspicuous species which grows on coquina rock or in habitats similar to *V. ventricosa*. The form of its mature vesicles, the composition of its sap, and the method of growth in culture of the aplanospores, to which this organism readily gives rise, were described by Steward (1939).

In *Dictyosphaeria favulosa* the condition is somewhat different. A description of the organism is given by Børgesen (1913) and other accounts and illustrations of it are familiar, in the works of Oltmanns (1922), Taylor (1928), and Fritsch (1935). In relatively small plants a central sap-filled cavity is enclosed as in single vesicles of *Valonia*. In *Valonia* a single cellulose wall encloses a single multinucleate protoplasmic layer which surrounds the central sap-filled cavity. In *Dictyosphaeria* the sheath surrounding the central cavity is more complex. It encloses between an outer and inner membrane a large number of coenocytic vesicles, each somewhat smaller but otherwise not dissimilar to vesicles of *V. ocellata*. These grow, expand, and by mutual compression form a compact thallus bounded by a thick wall on its outer surface and a thinner wall on its inner surface. Multiplication of the vesicles in the thallus occurs by fragmentation of the protoplasts, by what Børgesen described as 'segregative cell division', and these new units (comparable to the aplanospores of *V. ventricosa*) grow, first within the old vesicle, and then, after rupturing it, they expand and by mutual compression add to the thallus. The individual coenocytic members of this complex structure are said to be united by hapteron cells. In old plants this complex sheath of vesicles breaks and sea-water freely enters the central cavity, but the individual coenocytic members remain intact.

All these organisms were examined by methods similar to those already described for *V. ventricosa*. The walls of *V. macrophysa* and *V. ocellata* seem not to have been examined by the electron microscope hitherto. *Dictyosphaeria favulosa* was briefly referred to by Wilson (1951) from observations on material collected by one of us (F. C. S.).

Valonia macrophysa Kuetzing

The observations on *V. macrophysa* are shown in Pl. XIV. The photograph at Pl. XIV (iv) shows the appearance of the outer wall of *V. macrophysa* which had been incompletely cleared of its amorphous material. The preparation shows parallel strands, relatively coarse because of their uncleared state, in three different directions. In any one lamella the expansion of the wall has apparently pulled apart the cellulose strands somewhat in the manner described for *V. ventricosa*.

Plate XIV (i) shows a well-cleared preparation showing strong parallelism of the cellulose fibrils, here clearly seen in two lamellae: they are, however, somewhat more open than in *V. ventricosa* (Pl. IX (i)).

Plates XIV (ii) and (iii) show the wall of *V. macrophysa* as seen in section: Pl. XIV (ii) cut with the section almost parallel to one strand direction which is obviously repeated at intervals through the thickness of the wall. Pl. XIV (iii) shows the detail of three lamellae in a cross-section, so cut that it was not parallel to any fibril direction. The figure shows parallel fibrils in two directions. While a third direction of fibrils may be seen, it is not as conspicuous as in *V. ventricosa*.

Apparently the wall of *V. macrophysa* is looser in texture than that of *V. ventricosa* but constructed on the same general plan.

Valonia ocellata Howe

Plate XVI (i) shows a section, probably slightly tangential, through a sporeling which formed naturally within a mature vesicle of *V. ocellata*. The outer tangled weft of cellulose fibrils is heavily impregnated with amorphous material, but, within it, the regular parallel arrangement of fibrils in the second wall layer, which had already formed in the globular sporeling, is evident. In Pl. XVI (ii) there is heavy impregnation of the first-formed wall with amorphous material, through which the fibrillar structure of the wall is beginning to be seen. Pl. XV (i) and (ii) reveal again the typical condition, as seen in well-cleared preparations of the first-formed wall of *V. ventricosa*, and they show for *V. ocellata* the more randomly oriented fibrils of the younger wall and the approaching parallelism in a somewhat older one. In Pl. XV (iii) and (iv) the very pronounced parallel orientation in the mature wall is visible in preparations in which two lamellae are clearly evident. In Pl. XVI (iv) the wall is in cross-section and four successive lamellae appear with their strongly oriented parallel strands all running in the same direction within the thickness of the section: between these lamellae with parallel strands, however, are two other lamellae with more diffuse strands in quite different directions. Again it appears that the mature wall is made up of successive lamellae in which the chain direction changes abruptly and the strand direction seems to be repeated in every fourth layer, though the third strand direction is notably weaker than in *V. ventricosa*.

After dissolving out the bulk of the amorphous material Pl. XVI (iii) represents, at a magnification of 14,000, individual cellulose fibrils which together comprise the coarser 'rope-like' strands to be seen in other illustrations. (Even the fine central strand in the illustration is clearly double so that the maximum thickness cannot exceed 200Å.)

Again the general plan of wall structure and development for *V. ocellata* is similar to that of *V. ventricosa*.

Dictyosphaeria favulosa (C. Agardh) Decaisne

The outer membrane of the thallus consists of very many layers (Pl. XVII (i)). Pl. XVII (iii) shows relatively thin septa separating individual coenocytic members of the coenobium. By direct transmission the outer wall can easily be shown to consist of successive lamellae each with strictly parallel fibrils

(Pl. XVII (iv)). In the cross-section shown at Pl. XVII (ii) successive lamellae have these fibrils running more or less parallel to the direction of the cut (at least 6 such lamellae are clearly visible in the illustration) and, between these, there is a sort of 'herringbone effect' produced by two other lamellae in which the fibrils run in different directions. Again, therefore, the tendency is to be noted that the fibrillar directions that are laid down in the 1st are reproduced in the 4th, 7th, 11th lamellae, &c., and those of the 2nd are reproduced in the 5th, 8th, &c. This would again be consistent with a shift of 120° between successive lamellae, and the measured angle between the two such directions shown at Pl. XVII (iv) is almost exactly 120° .

GENERAL DISCUSSION

The implications of this work and its relations to the existing literature (both classical and current) on the subject of wall formation in general and in the *Valoniaceae* in particular, may be discussed under the following series of topics.

Surface striations in the wall.

Structure in relation to the method of expansion.

Conclusions from the polarizing microscope and X-ray diffraction.

The shift in fibril direction during growth.

Relations between cytoplasm and wall.

Structural units in the wall

Surface striations in the wall and their significance

Many writers have commented on the microscopically visible surface striations which are visible on cell-walls in general and in the *Valoniaceae* in particular. This subject was carefully studied by Correns (1893), who found that many algae show this effect in two directions—one set of striations following a left-hand spiral around the cell and the other a right-hand spiral nearly at right angles to the first. In *Chamaedoris annulata*, *Valonia utricularis*, and *Dictyosphaeria favulosa* he found a system of striations with *three* different directions.

Nägeli believed that the striations were due to differences in water content in the cell wall, but Correns (1893) came to the view that they represent small folds in the lamellae of the wall. Preston and Astbury (1937) suggested that the microscopically visible striations in *Valonia* ran parallel to the submicroscopic fibrils, and Sisson (1941) illustrated the surface striations in two directions which were held to be parallel to the directions of the crystallites as revealed by X-rays.

It is very clear from the evidence of the electron microscope that the visible *striations follow gaps in the lamellae*; during volume-increase the fibrils in a given lamella become separated and this forms the striations which are necessarily parallel to the original cellulose strands; see Pl. IX (i) and Pl. XIV (i). Correns's original but long-neglected observations of *three* striation directions

in the plants named is therefore in accord with the evidence of this paper and is suggestive that the cellulose strands are also laid down in *three* directions. The early evidence of Correns is particularly interesting in view of Wilson's (1951) indication that he could observe *three* sets of striations in *Valonia* and *Dictyosphaeria*.

Apparently most writers on *Valonia* have been so preoccupied with the hypothesis of two quite different mechanisms of wall formation, by which fibrils are first laid down in a spiral path and then in a series of great circle meridians passing through well-defined 'poles' (see Preston and Astbury, 1937), that they have been led to neglect such evidence of a third fibril direction as they themselves have noted (loc. cit., p. 94).

Wilson goes much farther (loc. cit., p. 284). He regards the lateral 'pulling apart' of the parallel fibrils as proceeding to such an extent that a 'ladderlike network is produced' (loc. cit., Pl. XIII, Fig. 1). In our examination of the walls freshly obtained from living *Valonia* this extensive degree of separation is not so evident; possibly it was more conspicuous in Wilson's preparations because they were made from vesicles in which the wall, by long storage in formalin-sea water, had become partially macerated. In fact it was this partial maceration that enabled Wilson to obtain his notable evidence—the first of its kind—that he had isolated lamellae from *Valonia* with fibrils in a single direction.

In short the evidence from the microscopically visible striations, known since Correns (1893), seems to be consistent with the idea here developed that in the Valoniaceae there are three fibrillar directions, which occur in different lamellae, which tend to shift abruptly through approximately 120° when passing from one lamella to the next, and which tend to recur at regular intervals as the lamellae are laid down.

The structure of the wall in relation to the method of expansion

Classical concepts of wall structure and formation, associated with the names of Nägeli (quoted from Ostwald's Classic No. 227, 1928) and of Wiesner (1886), regarded the wall as composed of small crystalline particles. The wall was supposed to grow either by insertion of new particles between the old ones (intussusception) or by the deposition of new particles upon the old ones (apposition). In the light of modern knowledge of cellulose structure Frey Wyssling (1945) postulated that the long cellulose molecules are held together by 'Haftpunkte' which are loosened by the protoplasm or by auxins, &c., thus permitting the walls to become plastic and to expand. While in this 'plastic' state he has assumed that the fibrils of the wall can be displaced, producing a looser network, within which new strands may be deposited. Against this background it is necessary to see the facts of wall formation from the very first-formed wall of the aplanospore to the mature wall of the vesicle.

An important advance made in this paper is the detailed description of the first-formed wall on a naked protoplasmic surface of an unattached spore.

This wall consists of a sheet of randomly arranged fibrils embedded in amorphous material. The extent to which this new wall can behave as a plastic solid evidently will depend upon properties of the amorphous material and upon the ability of the fibrils to slip past each other rather than the make and break of hypothetical 'Haftpunkte', for which there is no visible evidence either in the 'primary' or the 'secondary' wall. Priestley's concept (1930, loc. cit., pp. 102, 132) of the wall of the meristematic cell in angiosperms as a more plastic than elastic solid is more compatible with the structure observed in the first wall of *Valonia*: a similar structure is also known in the primary wall in angiosperms (Mühlethaler, 1950 *a* and *b*). However, it is clear that even this type of wall cannot accommodate itself by plastic flow to the expansion which occurs, because there is clear evidence in the pictures here presented that it breaks and tears apart as growth proceeds. Even in the first of the secondary lamellae, which show well-oriented parallel strands, it can be seen that these 'pull apart' at their ends. Later the lateral separation of fibres along their length also leads to a more open network, but the important features to be considered in the adjustment of the wall to the enlargement of the vesicle seem to be:

- (a) Irregular breaks and jagged tears which occur in the cellulose sheet after this is laid down.
- (b) The effect of the amorphous substances which is particularly conspicuous in the wall of the sporeling—to enable adjustments to occur by permitting flow and slip.
- (c) The repeated laying down of new, continuous cellulose layers at the protoplasmic surface which maintain the inner layer intact as the older and outer ones are deformed, cracked, and even eventually 'sloughed off'.
- (d) The thickening and expansion of the wall of the vesicle which occur simultaneously (cf. Steward, 1939, loc. cit., p. 92).

This stands in contradistinction to the idea sometimes held to apply to the cells of flowering plants, namely that the cell first expands while 'primary' wall exists and it only deposits 'secondary wall' after expansion has ceased. Eames and MacDaniels in their 'Introduction to Plant Anatomy' regard the expansion of the cell and the thickening of its wall as occurring concurrently as they clearly do in the vesicle of *Valonia* (see, e.g., p. 35).

This sort of mechanism need occasion no surprise, either in relation to *Valonia ventricosa* or to the Siphonales in general. An early but outstanding contribution to this field was made by Noll (1890), who investigated the growth of the wall of *Caulerpa*. Noll stained the filaments of *Caulerpa* with Prussian blue and subsequently allowed them to grow for 37 days. New lamellae were deposited, but the colour remained in the old lamellae in the form of the insoluble iron salt and it could be regenerated simply by soaking the preparations with potassium ferrocyanide and hydrochloric acid. As a result of these events the areas of the old wall could be distinguished by their

blue colour from the areas of new wall which were colourless. Noll describes how the old wall remained as isolated patches over the surface of the newer wall. He also visualized clearly that the wall could be enlarged to a limited extent before this type of fracture occurs. These old observations are in full agreement with the observations here recorded.

The structure of the wall from the use of the polarizing microscope and of X-ray diffraction

The appearance of the wall of *Valonia* under crossed Nicols was first observed by Correns (1893), who also examined *Dictyosphaeria*. Under the polarizing microscope these plants show an effect which Preston much later (1931) described as a mosaic. In a single cellulose layer with parallel strands the major optical axis is parallel to the length of the strands. When layers with strands in different directions are superimposed the interpretation becomes complicated, and it is not really feasible to obtain a detailed picture of the submicroscopic structure of the whole vesicle by the use of the polarizing microscope alone.

The application of the techniques of X-ray diffraction by Astbury, Marwick, and Bernal (1932) and by Preston and Astbury (1937) are well known. This type of investigation applied to a *mature* vesicle could not yield evidence of the structure of the *first-formed* wall. The conclusion of Preston and Astbury (1937) is familiar and often quoted, namely (loc. cit., p. 96): '... the chains of one set of layers form a system of meridians to the wall, while those of the other set build a system of spirals closing down on the two poles defined by the meridians'. The important point here is that wall deposition is conceived to occur by two *fundamentally different* processes, namely, one which lays down meridians, the other which lays down spirals. It would now appear that the evidence for meridians was not conclusive. There seems little doubt in our minds that:

- i. The X-ray observations, being made upon very short lengths of the fibrils, probably did not enable Preston and Astbury to distinguish between a system of meridians and a second set of spirally wound fibrils crossing the first, probably at or near 120° .
- ii. The work of these authors did not establish the tentative observation they did make concerning a third fibril direction and they lacked the knowledge that the first-formed wall had randomly arranged fibrils.
- iii. These facts led to the assumption of a specific 'pole' at the apex of the vesicle, even though there is little microscopical justification for it. In fact the concept of 'poles' almost followed from the method of lines of latitude and longitude by which these authors oriented themselves on the surface of the vesicle studied.

No doubt all three of these factors contributed to the formulation of a definite wall structure for this plant that seems incompatible with the present observations. Nevertheless, Preston and Astbury's (1937) work greatly

advanced our knowledge of this subject; and the further advances now being made in the reinvestigation of this plant are due to the superiority of the electron microscope, over X-ray diffraction, for the investigation of such problems as these.

Accepting the conclusions here drawn means that once the organism commences to lay down cellulose in parallel spirally wound strands it continues to form spirals: it does, however, abruptly change their direction from time to time, but even the shift, though abrupt, occurs in a regular manner.

The cause of the changes in the direction of the fibrils

There are here some outstanding problems to be answered.

- i. What causes the vesicle which first lays down cellulose strands at random to do so in the very orderly fashion shown by the first-formed parallel strands?
- ii. Having established one orderly method of depositing cellulose in parallel strands, what prompts the cell to cease this abruptly and to deposit new layers with the strand in a different direction oriented at approximately 120° to that in the preceding layer?
- iii. What mechanism causes the cell by successive changes at 120° to lay down a wall in which the fibril directions recur at regular intervals, i.e. at every fourth lamella?

These are difficult questions, impossible to answer on the evidence available. One hypothesis might be as follows:

In the protoplasm itself, probably in its surface, cellulose crystallites are synthesized. (We can see no evidence to support Farr's (1949) idea that the cellulose fibrils are preformed in plastids from which they uncoil before they are added to the secondary wall.) Cellulose crystallites or micro fibrils then move into place or reach the surface of the protoplasm. If so, they must be oriented in directions that are determined by the protoplasm, though the process which binds them together in the wall must resemble a process of crystallization.

Van Iterson (1936a) applied to *Valonia* an idea derived from Strasburger (1882), Crüger (1855), and Dippel (1868), who concluded that the secondary thickenings, within the primary wall layers, are formed along the direction of protoplasmic streaming: indicating that the moving protoplasm directs the deposition and orientation of the cellulose strands. The difficulty in applying any such concept to *Valonia* is that, as yet, no one seems to have seen active protoplasmic streaming in the vesicle, though it has been seen in the hapteron cells (Steward and Martin, 1937, p. 94). Even so, the real problem would only be pushed back one step and would raise the question, Why should the direction of streaming pass through a regular sequence of changes in direction which could determine the wall deposition in the manner which has been described? Van Iterson (1936) postulated that the wall can stretch most easily in directions at right angles to the fibrils already laid down

and, in consequence but for reasons that are not clear, the direction of the streaming and of the deposition of the fibrils changes. In any event the regularity shown in our cross and surface sections would seem to demand an angle of 120° not 90° . It seems best, therefore, to record the remarkable sequence of changes in direction of the deposited fibrils of cellulose in the wall of *Valonia* and to recognize that the explanation is as yet unknown. The phenomenon is certainly without parallel in the process of crystallization.

Roelofsen (1951) sees the explanation of the different orientation of fibrils in the outer and inner layers of the cotton hair in the supposition that intussusception of new cellulose occurs into the inner layer, while expansion occurs, but not into the outer. This enables the fibrils of the inner layers to remain in very flat spirals, almost transverse to the fibres' length, while in the outer layers they are passively extended. An explanation on these lines, however, does not satisfy the requirements of *Valonia*.

Relation between cytoplasm and wall

These problems tend to foster the revived idea that wall and protoplasm may be more intimately connected than is commonly supposed (see reference to this subject by Wilson, loc. cit., p. 286).

The synthesis of the glucose-glucose links of cellulose can hardly be conceived apart from the intervention of the cytoplasm through its enzymes and whatever protoplasmic milieu makes the reaction go in the intact cell whereas, at present, it will not proceed *in vitro*. The parallel of starch formation by phosphorylase from glucose-1-phosphate, and which is no longer regarded as absolutely dependent upon the living plastid, is relevant here. There is, however, no evidence for any more intimate connexion between wall and protoplasm than arises from the fact that it is only at the surface of the latter that the former develops. In other words, even a growing wall is not itself alive.

It is interesting to speculate whether the right enzymic machinery (comparable to phosphorylase and glucose-1-phosphate in starch formation) and a suitable configuration of surface (comparable to the grains of starch used to 'seed' the synthesis) could permit cellulose to be chemically produced. In fact one of us (Mühlethaler, 1950c) claims to have shown that the polymerization in cellulose synthesis in *Bact. xylinum* is enzymatically produced. Preston and Kuyper (1951, p. 253) go even further and interpret certain local accretions on their preparations as 'islands' of cytoplasmic material attached to the 'micro fibrils' of the inner wall layers. Preston and Kuyper (as they indeed recognize) worked with old vesicles subjected to rather crude killing and fixing. It is difficult for us, after examining critically fixed and growing vesicles, which show so emphatically the admixture of amorphous material, particularly in the first-formed wall layers, to regard such observations as meaningful in terms of cytoplasmic organization. Even after drastic chemical clearing it is difficult to obtain preparations in which the fibrils are completely free from adherent amorphous material, which we regard as pervading the

wall substance but not as the surface organization of the cytoplasm itself. Also our experience is that all the lamellae formed after and within the first randomly oriented one show markedly parallel strands: and random arrangements never recur after the parallel strands have commenced to form.

To interpret the way in which the long fibrils are finally arranged in such strikingly parallel fashion, from whatever crystallites the protoplasm forms, it may be necessary to have recourse to such ill-defined 'long-range' forces that are sometimes invoked to explain such phenomena as the pairing of relative large bodies like chromosomes or the aggregation of the long cells which can move together and form nerve-fibres even under tissue-culture conditions (Weiss, 1941).

Wilson (1951) clearly visualizes that any directive forces which cause the regular deposition of cellulose exist within the surface of the protoplasm itself, much as though it acted as a 'template' against which the crystallization occurs. Since nothing definite is known about this mysterious problem further speculation is hardly profitable. Of interest are the model experiments of Stuart (1943).

Structural units in the wall

The chemical unit of which cellulose is built is glycopyranose combined by 1-4 β -glucoside links. X-ray diffraction methods are interpreted to mean that the unit lattice in various natural fibres has the dimensions 10.3 Å, 7.9 Å, 8.35 Å, where the 10.5 Å spacing is at right angles to the fibre axis (Meyer and Misch, 1937).

The size of the most minute strands that have been resolved in this work is, therefore, interesting. In Pl. XVI (iii) their diameter is of the order of 200 Å; i.e. comprising some 20-25 unit lattices. They are thus somewhat comparable to the diameter (100 Å) of the 'micro fibrils' of Preston and Kuyper (1951, p. 251). In length, however, these structures appear to be potentially endless—like string wound around a ball of twine.

SUMMARY

1. The cell walls of various members of the Valoniaceae, *V. ventricosa*, *V. macrophysa*, *V. ocellata*, and *Dictyosphaeria favulosa*, have been investigated by the aid of the electron microscope.

2. Attention has been given to the structure of the wall by direct transmission, i.e. as seen in surface view, and in cross-section, and also to the changes which occur in the development of the wall at a naked protoplasmic surface as furnished by aplanospores or by the products of 'segregative cell division' as described by Børgesen.

3. The first cellulose laid down at a new surface, as on the aplanospores, consists of a 'two-dimensional' tangle of randomly arranged cellulose fibrils. These are embedded in amorphous material, which may be pectin. Even this first cellulose layer, however, cannot grow enough by plastic flow, or

stretch elastically, for it is eventually torn and a new cellulose layer is formed within.

4. Beneath the first-formed cellulose layer and the amorphous material cellulose lamellae are laid down. At first the strands of these layers are not exactly parallel, but they eventually acquire a very high degree of regularity.

5. The cellulose is laid down in successive lamellae in which the fibril direction shifts abruptly from lamella to lamella. The direction of the strands in successive lamellae is turned through 120° . Three such lamellae constitute a 'period' or 'stanza' of wall formation. Strand directions are, therefore, repeated in the 1, 4, 7, . . . $(1+3n)$ layers and in the 2, 5, 8, . . . $(2+3n)$ layers, &c.

6. The general pattern of wall structure and formation seems to be the same in the various plants (*V. ventricosa*, *V. macrophysa*, *V. ocellata*, and *Dictyosphaeria favulosa*) investigated.

7. The bearing of these results upon the interpretation of the growth of the cell-wall is discussed and is related to the other literature in the field.

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DESCRIPTION OF PLATES

Illustrating F. C. Steward's and K. Mühlethaler's paper on

'The Structure and Development of the Cell-Wall in the Valoniaceae as revealed by the Electron Microscope'

PLATE IX

Wall of *Valonia ventricosa*—mature vesicles.

- (i) Examined by direct transmission (wall as seen in surface view) mucilaginous material incompletely removed, three lamellae with directions at approximately 120°.
- (ii) As above: mucilaginous material incompletely removed.
- (iii) Wall in cross-section, showing many lamellae with fibrillar directions tending to be repeated in every fourth lamella.

PLATE X

Wall of *Valonia ventricosa*—aplanospores.

- (i) Surface of aplanospores showing amorphous material.
- (ii) First-formed wall of aplanospores cleared to show random arrangement of fibrils.
- (iii) Mucilaginous and cementing material on randomly arranged fibrils.
- (iv) Expansion of tangled weft of fibrils showing that they pull apart.

PLATE XI

Wall of *Valonia ventricosa*—aplanospores.

Various stages showing different degrees of order and parallelism, increasing from (i) to (iv), and the origin of a second lamella. Pulling apart of parallel strands as expansion occurs is shown in (iv).

PLATE XII

Wall of *Valonia ventricosa*—aplanospores.

Preparations cleared showing net-like wall open at base and with areas in which the wall is of varying degrees of density.

PLATE XIII

Valonia ventricosa: aplanospores at 15 hours.

- (i) Section showing wall in cross-section.
- (ii) First-formed wall, with loose net of randomly arranged fibrils seen in surface view.
- (iii) Wall in section showing a double layer separated by amorphous material.
- (iv) Outer layer of wall broken during expansion.
- (v) Whole sporeling with dense chloroplasts and several lamellae already laid down.
- (vi) Outer loose net of random fibrils with later formed lamellae within.
- (vii) Two well-defined layers with amorphous material between. Outer layer showing loose net of random fibrils, inner lamella impregnated with mucilaginous material.

PLATE XIV

Valonia macrophysa: wall of mature vesicles.

- (i) Cleared preparation showing wall in surface view.
- (ii) Cleared preparation showing wall in cross-section; section parallel to one strand direction.
- (iii) Cross-section in a direction not parallel to any strand direction, showing fibrils with different orientation in successive lamellae.
- (iv) Surface view of outer layers of mature vesicle, uncleared and showing remnants of lamellae which have pulled apart.

PLATE XV

Valonia ocellata: wall in surface view.

- (i) Cleared preparation showing an early stage of wall with random arrangement of cellulose fibrils.
- (ii) Later stage with greater tendency to parallelism.
- (iii) and (iv) Mature wall, cleared showing distinct lamellae with strands in different directions.

PLATE XVI

Valonia ocellata.

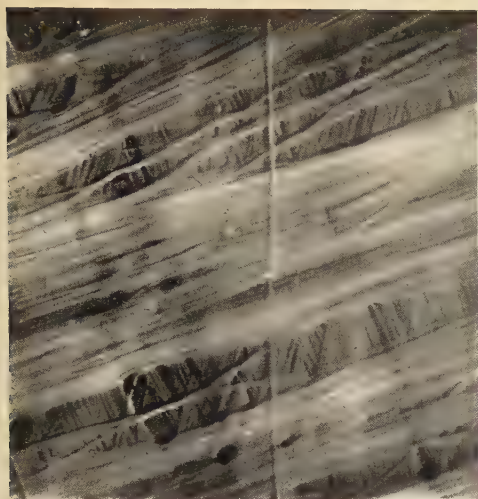
- (i) Section through whole aplanospore showing outer, randomly arranged wall impregnated with mucilage and inner wall with parallel strands.
- (ii) Surface view of wall of sporeling showing granular material with some fibrils visible.
- (iii) Individual strands separated by clearing with 6 per cent. HCl.
- (iv) Wall in cross-section after clearing, showing strands in different directions in successive lamellae.

PLATE XVII

Dictyosphaeria favulosa.

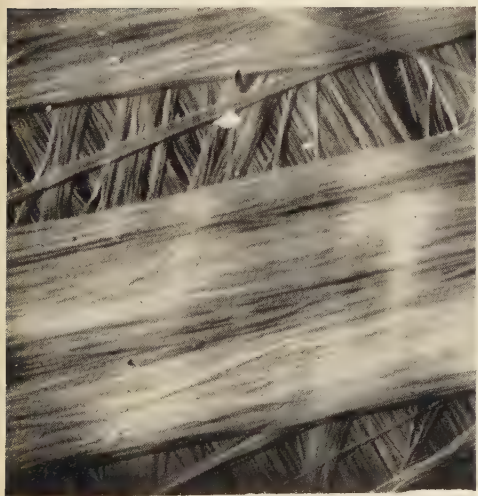
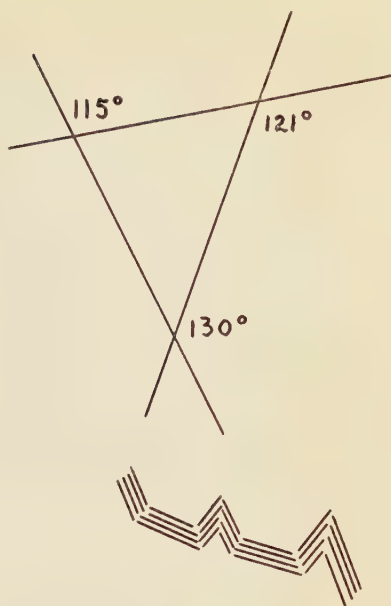
- (i) Many-layered outer wall of the colony (coenobium) shown in cross-section.
- (ii) Cross-section of the outer wall showing different strand directions in successive layers.
- (iii) Thin transverse septa between vesicles in the coenobium.
- (iv) Surface view of outer wall of colony showing strands crossing at an angle of 120° .

(The magnifications recorded are those of the original Plates which have been reduced somewhat to permit reproduction.)



i

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ii

13000×

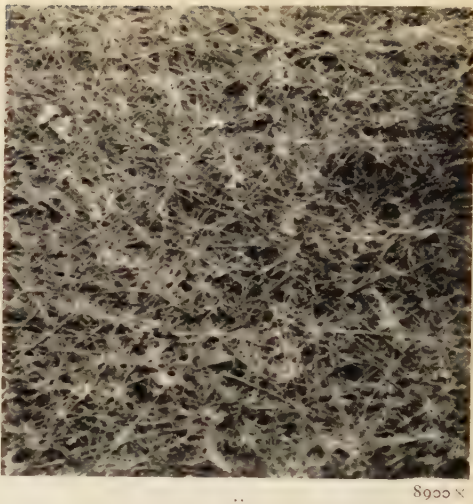


iii

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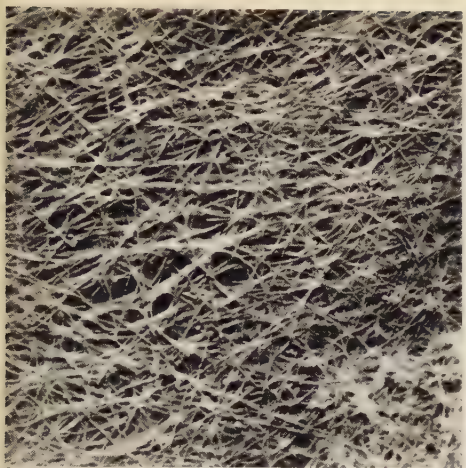
Valonia ventricosa

F. C. STEWARD and K. MÜHLETHALER



Valonia ventricosa

F. C. STEWARD and K. MÜHLETHALER



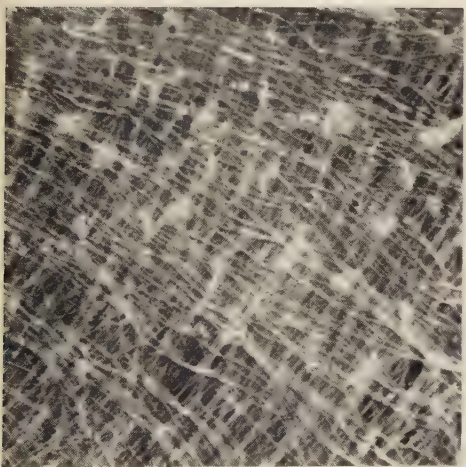
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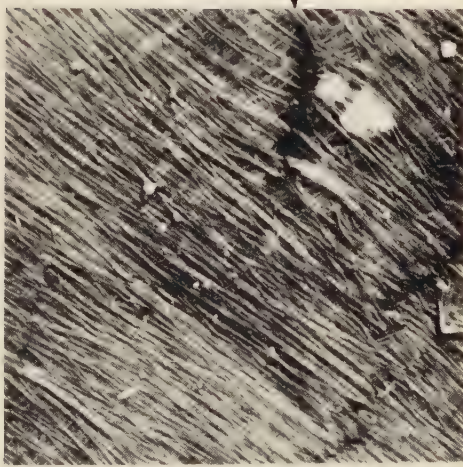
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9000 X



iii

6000 X



iv

9000 X

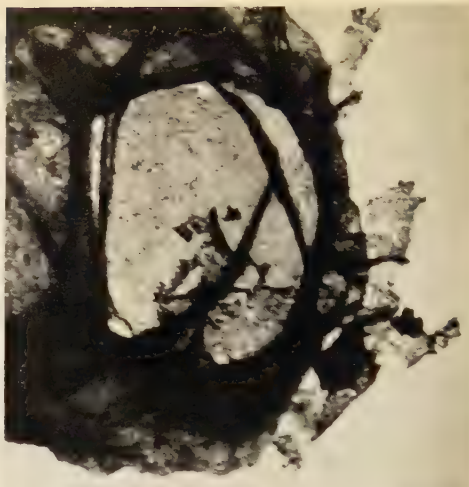
Valonia ventricosa

F. C. STEWARD and K. MÜHLETHALER



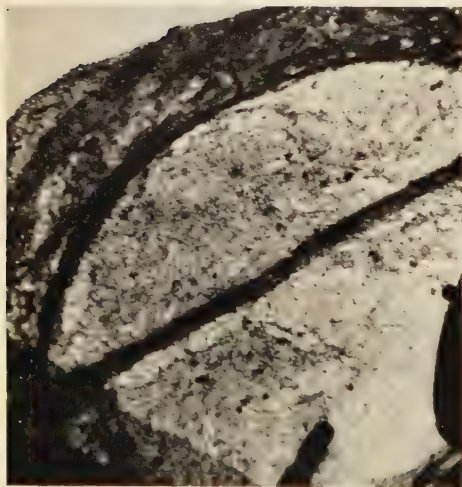
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i



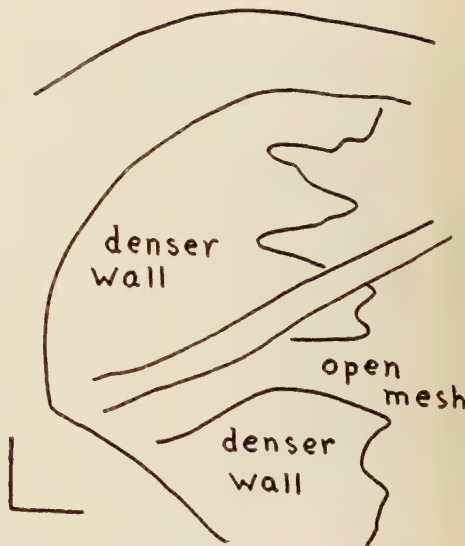
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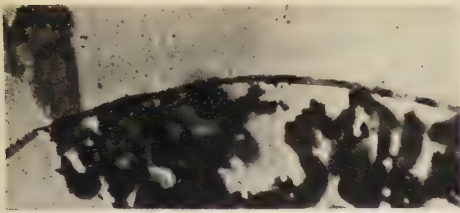
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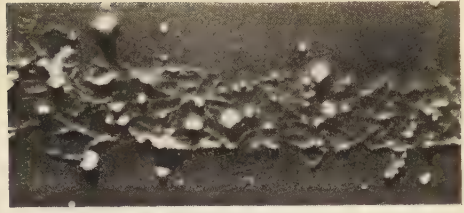
Valonia ventricosa

F. C. STEWARD and K. MÜHLETHALER



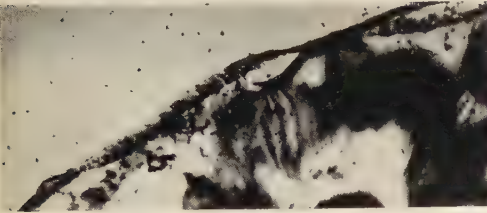
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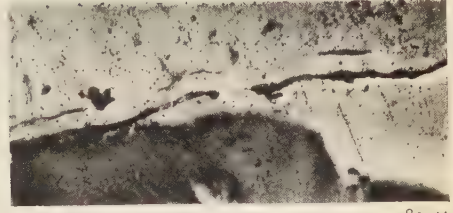
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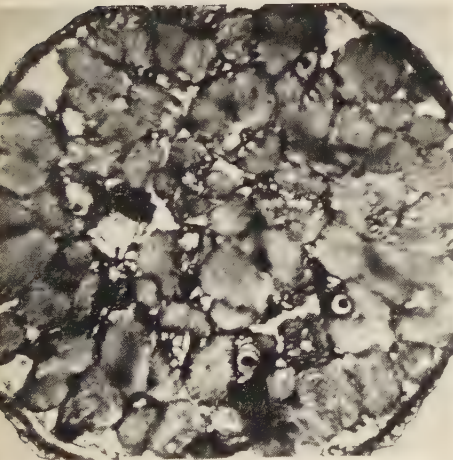
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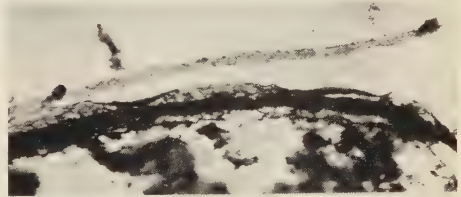
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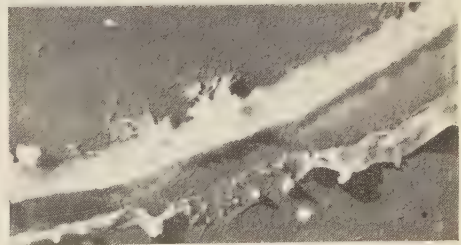
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2750 ×



vi

4800 ×



vii

8300 ×

Valonia ventricosa

F. C. STEWARD and K. MÜHLETHALER



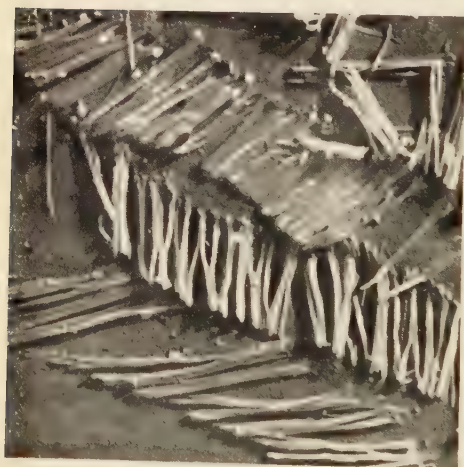
i

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iii

10800x



iv

8400x

Valonia macrophysa

F. C. STEWARD and K. MÜHLETHALER



i

5450×



ii

5600×



iii

8200×



iv

8300×

Valonia ocellata

F. C. STEWARD and K. MÜHLETHALER



i 8500 ×



ii 935 ×



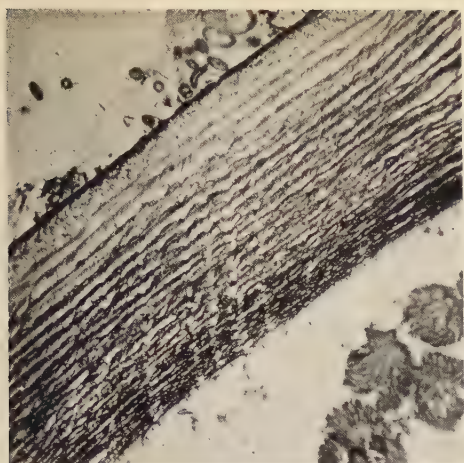
iii 10900 ×



iv 5900 ×

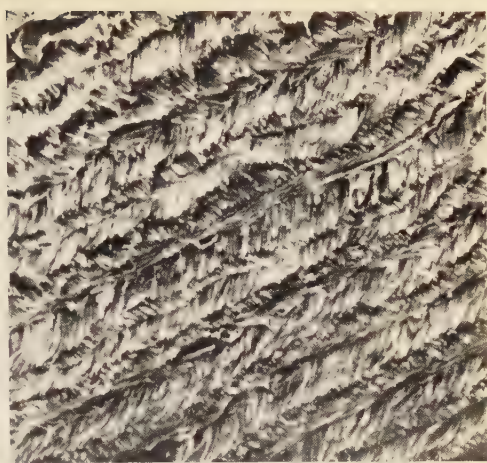
Valonia ocellata

F. C. STEWARD and K. MÜHLETHALER



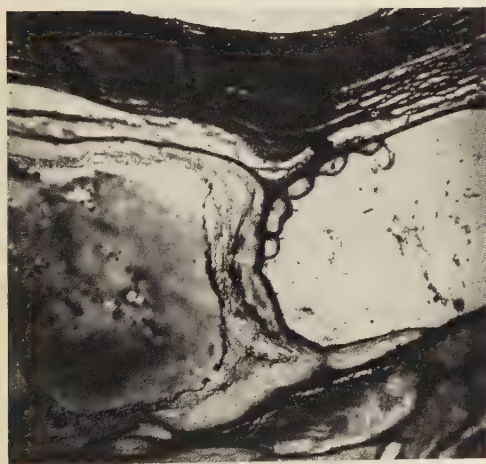
i

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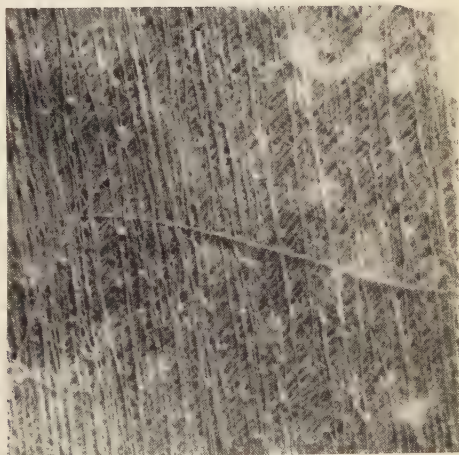
ii

6200×



iii

1550×



iv

5830×

Dictyosphaeria

F. C. STEWARD and K. MÜHLETHALER

Nickel and Cobalt Toxicities in Oat Plants

BY

ORNELLA VERGNANO

AND

JAMES G. HUNTER

(*The Macaulay Institute for Soil Research, Craigiebuckler, Aberdeen*)

With Plate XVIII

ABSTRACT

The types of symptoms and anatomical changes produced in oat plants by the addition of nickel and of cobalt to nutrient solution or by iron deficiency have been compared with those occurring in oat plants from several areas of Aberdeenshire and shown to be similar. Nickel contents are comparable whilst the cobalt content of the sand-culture plants is considerably higher.

INTRODUCTION

IT is generally recognized that relatively small concentrations of nickel and cobalt are toxic to plants. Investigation has shown, for example, that in nutrient solution approximately 2–40 p.p.m. nickel are toxic to beans and maize (Haselhoff, 1893), 2–60 p.p.m. to buckwheat (Cotton, 1930), 3–300 p.p.m. to oats, &c. (Scharer and Schropp, 1933), 2–15 p.p.m. to barley and beans (Brenchley, 1938), 0·5–5 p.p.m. to flax, &c. (Millikan, 1947*a*, 1948), and 15–30 p.p.m. to oats, sugar beet, &c. (Hewitt, 1948*a*, *b*). Cobalt also has been proved toxic, though with equivalent concentrations the effect is usually considered to be less than that produced by nickel; in nutrient solution maize and beans were affected by approximately 2–8 p.p.m. (Haselhoff, 1895), oats, &c., by 3–300 p.p.m. (Scharer and Schropp, 1933), wheat by 6–590 p.p.m. (Singh and Prasad, 1936), barley and beans by 1–15 p.p.m. (Brenchley, 1938), flax, &c, by 0·5–5 p.p.m. (Millikan, 1947*a*, 1948), and oats, sugar beet, &c., by 15–30 p.p.m. (Hewitt, 1948*a*, *b*).

The toxic symptoms produced by several heavy metals, including nickel and cobalt, have usually been described as being of two types: (*a*) specific effects characteristic of the metal involved, and (*b*) chlorosis caused by induced iron deficiency (Gile, 1916; Wallace and Hewitt, 1946; Hewitt 1946*a*, 1947, 1948*a*, *b*, and *c*; Millikan, 1947*a*, *b*, 1948; Morris and Pierre, 1949; Nicholas, 1950; Hunter and Vergnano, 1952*a* and *b*).

Excessive nickel affects oat plants in much the same way as excessive cobalt (Scharer and Schropp, 1933; Hewitt, 1948*b*), both treatments producing

white longitudinal striping or bleaching of chlorotic leaves. Analysis of plants and soils from certain areas in Aberdeenshire where similar symptoms were observed (Hunter and Vergnano, 1952) indicated that they were more likely to be the result of excessive nickel than of excessive cobalt, though the cobalt concentrations were also greater than normal. Control plants for comparison were obviously the first essential, and accordingly investigations were carried out to determine precisely the effects of nickel and cobalt on oat plants grown under controlled conditions. In these investigations, which are described below, leaf anatomy was studied, symptoms observed, and nickel and cobalt concentrations determined in the leaves of the plants. Because of the relation between toxicity and iron deficiency, the leaf anatomy of iron-deficient plants was also examined.

NICKEL TOXICITY IN OAT PLANTS

Experimental methods. Oat seeds (variety 'Victory') were sown in coarse quartz sand (13 per cent. held by a 14-mesh and 99 per cent. by a 30-mesh British Standard sieve) in 9 in. clay pots coated with bituminous paint. Water was supplied until the shoots were about $\frac{1}{4}$ in. high; thereafter adequate supplies of nutrient solutions were given daily, preceded by water flushing. Ten nutrient solutions were used, and these were prepared by diluting measured volumes of stock solutions to a predetermined volume; the composition per litre of the stock solutions (and volume required per 10 litres of each nutrient solution) were: NaNO_3 —250 g. (15 ml.); $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ —50 g. (20 ml.); K_2SO_4 —90 g. (20 ml.); $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ —430 g. (15 ml.); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ —250 g. (15 ml.); ferric citrate—6.40 g. (10 ml.); $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ —0.98 g.; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ —0.04 g.; H_3BO_3 —0.35 g. (10 ml.). The basic composition (p.p.m.) of the nutrient solutions was therefore: nitrogen, 138; phosphorus, 8.7; potassium, 81; calcium, 110; magnesium, 37; iron, 1.2; manganese, 0.24; copper, 0.01; boron, 0.06. Further supplies of iron and trace elements were available from the sand used. The pH of each solution was adjusted by sulphuric acid to 5.5, but a gradual increase to about 6.5 occurred when the solutions were in contact with the plant roots. The amount of nickel differed in the various solutions, the concentrations being 0.1, 1.5, 2, 2.5, 5, 10, 15, 20, and 30 p.p.m.

Some authors, for example (Brenchley, 1938), consider that the degree of toxicity produced by nickel and other trace elements depends upon the salts used. It is difficult to find justification for this theory as salts present in low concentration in nutrient solutions are usually considered to be absorbed in the ionic form. Experiments carried out by the writers showed nickel sulphate and nickel chloride in nutrient solutions of the above basic composition to be equally toxic to plants; in these experiments the nickel solutions were prepared from the same batch of nickel hydroxide to ensure that the amount of nickel present was identical in each case, whilst differences in contamination, such as might occur even from 'pure' chemicals, were reduced to a minimum. In the investigation described below, nickel was supplied as nickel sulphate.

The plants were observed and symptoms noted until 22 days after germina-

tion of the seed, by which time the plants receiving nutrient solutions containing 10 p.p.m. (or more) nickel were stunted; these plants were therefore sampled, and to ensure continuity of results some of the plants receiving 0, 2.5, and 5 p.p.m. in their nutrient solutions were also sampled. The remainder of the plants receiving less than 10 p.p.m. nickel were grown for a further 11 days before being sampled.

Results. With relatively low concentrations of nickel in the nutrient solution (1 or 1.5 p.p.m.) a slight mottling appeared at the tip of the first leaves about 10 days after germination (that is, about 8 days after receiving the solution containing nickel). Within 12 hours this mottling was replaced by longitudinally striped colourless (necrotic) areas. These gradually extended, and similar areas appeared over the middle and upper parts of these leaves. While the necrotic areas were extending in the first leaves the second leaves were developing; these usually remained normal, though slight chlorosis and necrosis sometimes occurred. The young leaves which developed later were normal or almost normal.

With higher nickel concentrations (2, 2.5, or 5 p.p.m.) the first leaves became mottled, and subsequently (within a week of germination) longitudinally striped with completely colourless areas; the development of the symptoms was as described above. The leaves which developed later were generally not so wide and the chlorosis was diffuse, though occasionally intervenal. Longitudinal white areas appeared simultaneously in the upper and mid-leaf regions of these leaves and spread slowly to the lower parts and more rapidly towards the margins, so that the leaves became completely devoid of pigment in some parts; the green areas where pigment was retained alternated with the white and were usually diffusely chlorotic though sometimes almost normal.

With the higher nickel concentrations (10, 15, 20, and 30 p.p.m.) the growth of the plants was considerably reduced. The first leaves were narrower and weaker, whilst the second and third remained rolled and stunted. The symptoms developed as described above and extended very rapidly, the leaves becoming completely colourless and eventually dying.

Roots appeared to be unaffected by concentrations of nickel up to 2 p.p.m.; 2.5 and 5 p.p.m. nickel led to a reduction in growth and in formation of secondary roots; roots were fibrous and of a dark ochraceous colour. With higher concentrations root development was very poor, levels of 20 and 30 p.p.m. producing only a few dark brown roots with no secondary rootlets.

From these and other experiments it was noted that, although chlorosis always preceded necrosis, the necrotic areas in older plants were sometimes completely surrounded by tissue of a normal green colour; that is, when necrosis developed it did not necessarily occur in chlorotic leaves.

The chlorosis which usually developed in the leaves of plants supplied with nickel was found to respond, especially in the young stages, to painting with a 0.25 per cent. solution of ferrous sulphate containing 0.025 per cent. sodium lauryl sulphate as a wetting agent—indicating that the condition was due to iron deficiency.

The concentrations of nickel in the dry matter of fully expanded leaves from plants in the experiment were determined and the results are recorded in Table I. These results show that the uptake of nickel from the nutrient solutions was very marked even where the concentration of nickel in the nutrient solution was relatively low.

TABLE I
Nickel Content of Oat Leaves

Nickel concentration (p.p.m.) in solution.	Nickel concentration (p.p.m.) in fully expanded leaf dry matter	
	1st sampling	2nd sampling
0	7	6
1.5	—	86
2.5	66	116
5	88	160
10	144	—
15	370	—
20	590	—
30	925	—

The plants receiving 2.5 and 5 p.p.m. nickel were similar to moderately and severely affected plants from the Aberdeenshire areas and the nickel contents were of the same order:

		Nickel concentration (p.p.m.) in fully expanded leaf dry matter.
Plants at same degree of maturity	{ Field sample moderately affected	99
	{ Field sample severely affected	134
	{ Sand culture (2.5 p.p.m. nickel)	116
	{ Sand culture (5 p.p.m. nickel)	160

Comparison of superficial leaf symptoms and of leaf composition therefore supported the diagnosis that the abnormal plant growth associated with certain Aberdeenshire soils is due to excessive nickel.

COBALT TOXICITY IN OAT PLANTS

The effect of cobalt on oat plants was examined as described above for nickel, the cobalt levels in the nutrient solutions being 0, 5, 10, 15, 20, and 50 p.p.m. as cobalt sulphate.

The experiment was continued for 35 days after germination of the seed; symptoms were noted, and fully expanded leaves from some of the treatments were sampled on completion of the experiment. As in the case of nickel, both chlorosis and necrosis developed, the appearance of the plants at the time of sampling being briefly described in Table II.

Painting the chlorotic areas which were formed, especially in the young stages, with a solution of ferrous sulphate containing sodium lauryl sulphate, indicated that in this case also the condition was due to iron deficiency.

TABLE II
Effect of Cobalt on Oat Plants

Cobalt concentration (p.p.m.) in nutrient solution	Description of plants
0	Plants normal.
5	Plants slightly chlorotic (chlorosis intervenal) and occasionally necrotic (necrosis white, striped, and confined to first leaves). Roots slightly smaller than control.
10	Plants moderately chlorotic (chlorosis markedly intervenal) and occasionally necrotic (necrosis white, striped, and more conspicuous than with 5 p.p.m. cobalt). Roots small and fibrous.
15	Plants small and severely chlorotic (chlorosis intervenal) and more necrotic than with 10 p.p.m. cobalt (necrosis affecting first leaves and also some younger). Roots small and fibrous.
20	Plants small and severely chlorotic (chlorosis intervenal) and more necrotic than with 15 p.p.m. cobalt (necrosis affecting first leaves and also some younger). Roots small and fibrous.
50	Plants stunted and severely necrotic (necrosis affecting first and later leaves almost completely); some red-coloured leaf-tips. Roots stunted, fibrous, and ochraceous.

Whilst cobalt resembled nickel in that it produced both chlorosis and white necrotic areas, the symptoms of cobalt toxicity were distinctive: (*a*) the chlorosis was prominently intervenal, whereas with nickel it was usually diffuse; (*b*) the necrosis followed the chlorotic pattern occurring in narrower stripes than with nickel; and (*c*) the necrosis developed more slowly than with nickel, and more slowly than the general toxicity, so that a relatively low degree of necrosis was associated with poor plants with narrow leaves.

The cobalt contents of fully expanded leaves of plants from the treatments sampled were determined with the following results:

Cobalt concentration (p.p.m.) in nutrient solution.	Cobalt concentration (p.p.m.) in fully expanded leaf dry matter.
0	0.8
5	116
15	640

The level of cobalt in the plants was therefore high, even where the degree of necrosis was low, and considerably higher than the 6 p.p.m. found in the leaves of comparable plants from the affected areas. It is therefore improbable that cobalt toxicity is a factor contributing to the infertility of these soils.

EFFECT OF NICKEL AND COBALT TOXICITIES AND OF IRON DEFICIENCY ON THE LEAF ANATOMY OF OAT PLANTS

This investigation was undertaken to determine the differences in leaf anatomy associated with symptoms of nickel toxicity, cobalt toxicity, and iron deficiency and with the symptoms produced by an affected soil.

Experimental. Oats were grown in (a) a soil-sand mixture (two soils, one treatment each) and (b) sand (five treatments).

One soil was a peat from an affected area (Hunter and Vergnano, 1952) and the other an ordinary fertile loam known to produce normal plants and therefore suitable as a control. Seeds were sown in a mixture of 7 parts soil and 2 parts coarse quartz sand contained in 9-in. clay pots coated with bituminous paint.

The sand-culture treatments were carried out as described previously except that (a) the sand used was purified by successive treatments with a 15 per cent. hydrochloric acid and 0.5 per cent. oxalic acid solution (Hewitt, 1946b), the adsorbed hydrogen ions (McHargue, 1923) being displaced by leaching intermittently for 4 days with the nutrient solution to be used, and (b) the nutrient solutions were supplied from the time of sowing. The basic composition of the solutions used for sand culture was as given previously except that iron was omitted where appropriate; five solutions differing in nickel, cobalt, or iron concentrations were supplied to the plants, the essential differences between the solutions being shown in Table III. Nickel and cobalt were supplied as sulphates and iron as ferric citrate.

TABLE III
Essential Differences in Nutrient Solution Composition

Solution	Concentration (p.p.m.) in nutrient solution		
	Ni.	Co.	Fe.
Control	—	—	1.2
Nickel (2.5 p.p.m.) toxic . . .	2.5	—	1.2
Nickel (5 p.p.m.) toxic . . .	5.0	—	1.2
Cobalt toxic	—	15	1.2
Iron deficient	—	—	—

All pots (for both soil and sand culture) were sown at the same time.

The oats receiving 5 p.p.m. nickel and those growing in the abnormal soil were sampled at intervals until definite white areas appeared in the first leaves—at that time incompletely expanded; these plants developed symptoms of the same type and at the same rate, but the symptoms were more widespread in the leaves of the plants in the abnormal soil. The plants receiving 2.5 p.p.m. nickel were also sampled at these times; at the final sampling the leaf apices of these plants were mottled, but necrosis appeared the following day. Symptoms developed much more slowly in the plants receiving the cobalt-toxic and iron-deficient solutions, and sampling dates were correspondingly later; the leaves of the cobalt plants were markedly chlorotic (chlorosis intervenal) and very slightly necrotic at the final sampling, whilst those of the iron-deficient plants were yellow and parchment-like in appearance. The age of and symptoms in the plants when sampled are given in

Table IV. Both soil and sand-culture control plants were sampled at the same stages as the other plants.

TABLE IV
Data on Plants sampled for Anatomical Investigation

Nickel-toxic Sand Culture and Abnormal Soil

No. of days after germination	Appearance of plants	
	2.5 p.p.m. Ni.	5 p.p.m. Ni. and abnormal soil
2	Coleoptile: normal	Coleoptile: normal
4	Coleoptile: normal	Coleoptile: normal
7	First leaf: normal	First leaf: slightly chlorotic
9	First leaf: slightly chlorotic	First leaf: apex mottled
12	First leaf: apex mottled	First leaf: apex necrotic

Cobalt-toxic and Iron-deficient Sand Cultures

No. of days after germination	Appearance of plants	
7	First leaf: normal	
15	First leaf: chlorotic	
21	First and second leaves: very chlorotic and (cobalt plants) slightly necrotic.	

The coleoptiles (whole at the first sampling and subdivided into 5–6 mm. pieces at the second) were fixed in La Cour's 2BE fluid. Leaves were cut into similar pieces and fixed in formalin-aceto-alcohol. After embedding the material in paraffin, sections (10–12 μ) were cut with a Cambridge rocking microtome, stained with safranin and Delafield's hematoxylin, and mounted in Canada balsam.

Control plants. The sections of control plants grown in soil and in sand were essentially the same at each sampling, and structure of the leaves of control plants remained constant from the 7th to the 21st day after germination. A typical leaf section (Pl. XVIII, Fig. 1) can therefore be used as control for leaf sections of abnormal plants (sampled between 7 and 21 days after germination) from either soil or sand cultures.

Plants receiving excessive nickel. The following description is of the anatomical changes in the leaves of plants receiving 5 p.p.m. nickel in the nutrient solution. The changes induced by 2.5 p.p.m. nickel were identical, though their rate of development was slower and the leaf area affected was smaller. Though sampling dates were separated by several days, it was possible to deduce the sequence of intermediate events from the differences in structure between leaf areas of different symptom degree. The information gained in this way has been incorporated in the description given below.

The leaves were normal in appearance for 6 days after germination and anatomically unchanged when examined on the 4th day. By the 7th day the apices of the first leaves were slightly chlorotic. In the chlorotic areas most

of the epidermal cells were normal; some were enlarged and distorted, but the majority were crushed and almost unrecognizable—particularly the pairs adjacent to the stomata where, as a rule, the crushing first became evident. In the mesophyll tissue—which normally consists of several cell layers and wide intercellular spaces—the cells were clumped together irregularly (though unaltered in shape or in number and position of the chloroplasts) and the intercellular spaces were consequently larger; this change was first apparent in the stomatal areas of the intervenal regions and was usually found associated with the damaged epidermal cells adjacent to the stomata. Fibrovascular bundles and sclerenchyma were unaltered.

Nine days after germination the leaf areas in the immediate vicinity of the vascular bundles were dark green in colour, whilst some intervenal areas were chlorotic and others mottled. In the chlorotic areas many cells were found with the chloroplasts agglutinated to one end, and a few had broken walls. In the mottled areas the number of broken cells was great and a small mass of undifferentiated tissue (presumably composed of protoplasm and disintegrated cell walls) was present (Pl. XVIII, Fig. 2).

Twelve days after germination the lysis described above had become the dominant feature, and its development was very rapid. Most of the mesophyll cells had disintegrated, the chloroplasts were absent—the area being completely devoid of green pigment—and between each vascular bundle was shrunk undifferentiated mass composed of tissue remnants; this condition was associated with the 'white necrotic' symptoms. At this stage the lysis extended to the vascular bundles and the epidermis was sunk in the mesophyll cavity. The mesophyll tissue surrounding the vascular bundles was often affected, but damage in the leaf margins was rare. The vascular bundles were affected, but to a much smaller extent than the mesophyll; the xylem was weak and brittle (and therefore difficult to section), the phloem contained inclusions, was heavily stained and sometimes necrotic, and, in the more severely affected cases, the cells of the bundle-sheath were compressed and flattened inwards.

The sequence of events appears to have been agglutination of the chloroplasts, bursting of cell-walls, formation of an undifferentiated mass of protoplasm and disintegrated cell-walls, and collapse of the epidermis.

Plants grown in abnormal soil. In these plants the anatomical changes were as described above (Pl. XVIII, Fig. 3). The rate of development of lysis, however, was more rapid (and consequently the collapse of the epidermis came more quickly), occurring almost simultaneously in all the intervenal regions and producing total necrosis of the areas. The xylem was more affected, becoming extremely thin, and the phloem was more necrotic with many inclusions and pronounced staining.

Plants receiving excessive cobalt. Symptom development was considerably slower with cobalt than with nickel and the degree of intensity at the last sampling date was much less (Pl. XVIII, Fig. 4). Though the anatomical changes occurring in the cobalt and nickel plants were similar, there were

several minor differences for which the difference in intensity of action was probably partly responsible. The anatomical changes peculiar to cobalt were:

- (a) The majority of the epidermal cells remained normal; of the abnormal cells only a few were swollen, most were crushed.
- (b) The mesophyll intercellular spaces were only slightly enlarged.
- (c) Breakdown of cells and massing of cell debris occurred in only a few areas.
- (d) The epidermis was never found more than slightly sunk.
- (e) The xylem was normal.
- (f) The phloem showed no trace of necrosis.

Iron-deficient plants. The symptoms of iron deficiency developed slowly in the plants and were first observed as a slight intervenal chlorosis on the 15th day after germination; this chlorosis soon became more generalized, that is, diffuse.

The pattern of anatomical change followed was essentially similar to that of nickel and cobalt—even to signs of the characteristic lysis. Differences were evident, however, and the degree of ultimate damage was considerably less. The principal differences in the iron-deficient plants were:

- (a) Very few epidermal cells were normal; the majority of the abnormal cells were swollen and only a few were crushed.
- (b) The intercellular spaces of the mesophyll were enlarged, particularly those below the stomata, but the normal distribution of cells was unchanged.
- (c) Many mesophyll cells contained large plastids highly stained by hematoxylin.
- (d) The cell-wall breakdown was found very occasionally—when chlorosis was very severe.
- (e) Large empty cavities into which the epidermis was slightly sunk were very occasionally found between the vascular bundles when chlorosis was very severe.
- (f) The cells of the bundle-sheath were generally swollen and occasionally (when chlorosis was severe) compressed and flattened inwards.
- (g) The lysigenous cavity at the base of the vascular bundle was generally very wide.
- (h) The xylem was normal.
- (i) The phloem was heavily stained, very rich in inclusions, and appeared to be slightly deformed.

CONCLUSIONS

The anatomical investigation revealed that nickel and cobalt in excess almost certainly have the same effect—any difference being merely in degree of activity—and that the chlorotic feature of the symptoms is very similar to iron-deficiency chlorosis. The character of the anatomical change associated

with severe iron deficiency indicates that necrosis induced by nickel or cobalt may be the result of a very severe, localized, iron deficiency; this, however, is not confirmed by the results of other experiments undertaken by the authors (Hunter and Vergnano, 1952*b*), which suggest that the necrosis is associated with interference in copper metabolism.

The development of chlorosis and subsequent appearance of necrosis in the mesophyllic area between the vascular bundles suggest that nickel is translocated in the conducting system and then moves to the areas of greater metabolic activity where it becomes concentrated. The widening of the intercellular spaces immediately below the stomata, and the collapse of the agglomerated cells of the mesophyll nearest to this region, indicates that in this area the nickel accumulation is probably greater than in the area immediately surrounding the vascular bundles. Evidence of this movement and accumulation is the concentration of nickel in young parts of oats rather than old, and in the grain, and in the leaf lamina of other plants as opposed to the leaf midrib (Hunter and Vergnano, 1952). In the vascular bundles the phloem showed some degree of necrosis at the stage when the mesophyll was disintegrated, indicating that some nickel is also translocated to the phloem; the lack of damage in the xylem is probably due to the absence of cytoplasm in this tissue.

Necrosis of tissue is not a characteristic peculiar to nickel toxicity—similar lytic processes have been described by Reed and Dufrénoy (1935) and Reed (1938) in connexion with deficiency and toxicity of zinc in a variety of plants (not including oats)—but its development in the case of nickel is outstanding in its severity and rapidity.

It is evident that the symptoms produced in oat plants by excessive nickel are anatomically of the same type as those found in the oat plants from the abnormal soils—which supports the conclusion already reached that nickel toxicity is operative in these areas.

SUMMARY

1. The symptoms produced by nickel and by cobalt in oat plants in sand culture are examined and are shown to be a combination of induced iron-deficiency chlorosis and necrosis; the symptoms are similar though not identical.

2. The symptoms produced on oat plants grown in sand culture with nutrient solutions containing 2.5 and 5 p.p.m. nickel are identical with or very similar to those in oat plants from several areas of Aberdeenshire. The nickel contents of fully expanded leaves of these plants are also comparable. The symptoms produced by cobalt in sand culture are similar to but never identical with those found in plants from the above areas, and the cobalt concentrations in the sand culture plants are considerably higher.

3. The anatomical changes produced in the leaves of oat plants by excessive nickel, excessive cobalt, deficient iron, and by the abnormal factor in the affected areas are examined and found to be similar though not identical.

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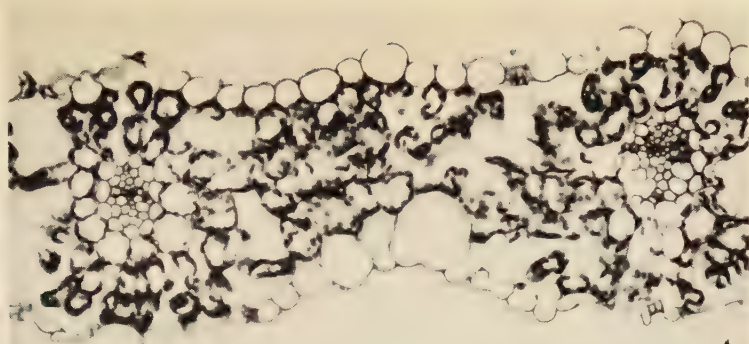
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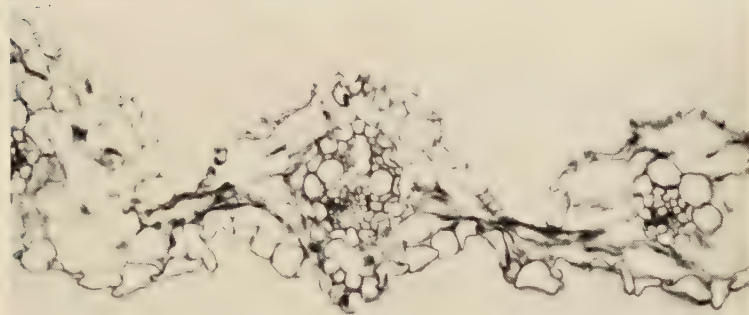
DESCRIPTION OF PLATE XVIII

Illustrating O. Vergnano's and J. G. Hunter's article on 'Nickel and Cobalt Toxicities in Oat Plants'

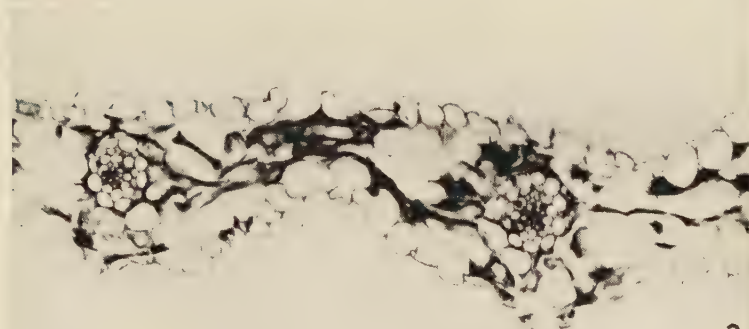
- Fig. 1. Leaf section of normal oat plant. ($\times 176$.)
- Fig. 2. Leaf section of oat plants receiving excessive nickel. ($\times 176$.)
- Fig. 3. Leaf section of oat plant from abnormal soil. ($\times 176$.)
- Fig. 4. Leaf section of oat plant receiving excessive cobalt. ($\times 176$.)



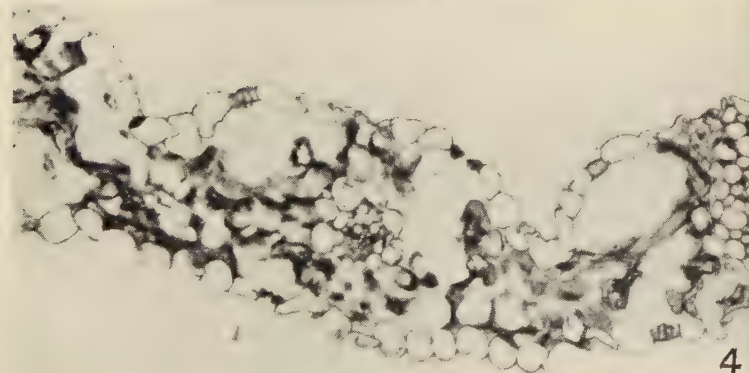
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The Mating System in *Saccharomyces*

BY

M. AHMAD¹

(Botany School, University of Cambridge²)

With three Figures in the Text

ABSTRACT

Many, but not all, *Saccharomyces* species are heterothallic. The mating types in heterothallic species are determined by two allelic genes. The mating-type genes occasionally mutate from one to the other. Vegetative cells of opposite mating type were found in some single ascospore colonies. They show conjugation tubes, stimulated by the proximity of cells of the other mating type. The cultures also show zygotes, which on sporulation yield *plus*(+) and *minus*(-) progenies.

The zygotes bud off diploid vegetative cells which grow faster than the original haploid cells and so tend to replace them. If mutation occurs early, replacement is complete and the culture gives no mating reaction but sporulates. If it occurs late, the culture is a mixture of haploid cells giving a mating reaction and diploid cells that will sporulate.

INTRODUCTION

SACCHAROMYCES cerevisiae has a simple life-cycle. Round haploid vegetative cells fuse to give elliptical diploid cells and the latter, under suitable conditions, turn into asci, each usually containing four ascospores. Each ascospore on germination founds a haploid colony in which the cells are round. The status of the mating system in yeast has, however, been a subject of acute controversy.

Sexuality in yeasts was established in 1900 by Hoffmeister, yet as late as 1931 (Guillermond, 1927; Stelling-Dekker, 1931) it was believed that occasionally the life-cycle could be continued by parthenogenetic ascospore formation in a haploid stock. However, Satava's (1918, 1934) work, followed by that of Winge (1935, 1944) and Winge and Laustsen (1937, 1938, 1939), showed there was always a regular alternation of haploid and diploid phases in the life-cycle. In no case were the ascospores formed during the haploid phase. Since Winge (1935) observed diploidization followed by normal ascospore formation in single spore cultures, he concluded that 'In these yeasts fertilization does not consist in a union of cells that differ genotypically . . .', and hence that they were all homothallic.

¹ Present address: Department of Biology, Dacca University, Ramna, Dacca, Eastern Pakistan.

² This work was done from 1945 to 1948 and was submitted for the degree of Ph.D. University of Cambridge.

Lindegren and Lindgren (1943 *a, b, c*, and *d*) mated the haploid cultures from the four spores of an ascus in all combinations and showed that two of the four spores belonged to one mating type and two to another. Normal conjugation with the production of a zygote occurred only between spores or cells of opposite mating types. They concluded that all species of *Saccharomyces* were heterothallic. However, they agreed with Winge that mating could occur between cells of presumably the same mating type, distinguishing this as illegitimate mating, the normal union between cells of opposite mating type being legitimate. They also considered that asci could be produced rarely by parthenogenesis in haploid stocks (1943 *c*).

The present work was begun with the object of testing whether species of *Saccharomyces* are homothallic or heterothallic, in the hope of resolving the differences existing between the conclusions of previous workers.

MATERIALS AND METHODS

The stocks of yeast used in this investigation were:

1. *Saccharomyces cerevisiae*, Danish Baking Yeast, from Distillers' Company Limited, Surrey.
2. *Saccharomyces cerevisiae*, Brewers' Yeast, from Professor Hopkins, University of Birmingham.
3. *Saccharomyces carlsbergensis* from Delft, Holland.
4. *Saccharomyces* sp. Form 237, from Professor Winge, Copenhagen.

Stocks of the yeasts 1 and 2 were kindly provided by Dr. M. Ingram of the Low Temperature Research Station, Cambridge, while number 4 was given by Dr. R. Davies of the Department of Biochemistry, Cambridge.

Two per cent. liquid malt was used for subculturing and testing for mating reaction. Asci were obtained either on nutrient agar slants or on gypsum blocks after growth on a modified form of the Lindgrens' solid presporulation medium (1944) of the following composition:

Beetroot and leaf extract	. 93.0 c.c.
Glycerine	. 2.5 c.c.
Glucose	. 3.0 g.
Dried yeast	. 2.0 g.
Agar	. 3.0 g.

The stock to be sporulated was spread on a slant of the medium and incubated at 25° C. for 2 days. Then 3 c.c. of sterile water were added and the tube kept in a slanting position for about 10 minutes. When the cells were thoroughly wetted, the tube was shaken vigorously and the suspension poured on a gypsum block stood in water acidified to pH 5.0 with acetic acid.

Since the latter method gave slightly greater proportions of sporulating cells and also larger asci it was used whenever any stock was to be analysed genetically. However, because of its simplicity, the first method was employed to test the sporing capacity of single-spore cultures.

Sporulation in some stocks that had lost their sporing capacity could be revived after three or four passages on Lindegren's presporulation medium and gypsum blocks.

For germination of the ascospores a medium recommended by Lindegren, Spiegelman, and Lindegren (1944) was used. It has the following composition:

Autolysed yeast extract powder	. 2.0 g.
Ammonium sulphate	. 1.0 g.
Potassium dihydrogen phosphate	. 1.866 g.
Calcium chloride	. 0.125 g.
Bacto peptone	. 3.0 g.
Potassium phosphate	. 0.133 g.
Magnesium sulphate	. 0.25 g.
50% sodium lactate	. 1.0 c.c.

When the medium was required for use, 4 per cent. glucose and 0.75 per cent. agar were added. The percentage of agar was low to provide a film of water on the surface of the drop of medium to which the spore was transferred for germination. Particles in the agar sometimes made it difficult to see whether the spore had been transferred to the drop of the germination medium. It was therefore cleared by means of egg albumen.

Crosses were made by mixing a loopful of cells 24–48 hours old from the two stocks in a tube of 2 per cent. liquid malt and incubating at 25° C. for 24 hours. Zygotes were then isolated before they began to bud and were grown separately.

I. MATING SYSTEM IN DANISH YEAST

(a) *Segregation of mating types.* The strain used formed four-spored asci abundantly, but in only one case were all four spores from an ascus germinated (Table 1, expt. 1). Cultures raised from each of them were mated in all possible combinations and it was found, in agreement with Lindegren, that two spores belonged to one mating type while two belonged to the other. When tested for sporulation capacity each of the four stocks was negative. The two which gave the strongest mating reaction were used in the subsequent work as standard testers, and were arbitrarily designated + and —.

When haploid cells of opposite mating type are mixed together, many of the cells are stimulated to produce conjugation tubes, so becoming pear-shaped. Pairs of stimulated cells, which are the gametes, fuse by their narrow ends to produce a dumbbell-shaped zygote. When the latter buds, the new cells are typically elliptical in shape and larger than the round haploid cells. Two cultures are recognized to be of opposite mating type if, in mixture, these events occur.

When progenies from five other single spores were tested, three gave a mating reaction and did not form any spores while two gave no mating reaction but did form spores. The two single-spore progenies which lacked any mating reaction but formed spores would appear to be homothallic, as Winge claimed for *Saccharomyces*. The first results thus gave some evidence

for the contentions of both Winge and Lindegren. The problem is how these observations may be reconciled.

TABLE I

Segregation of Mating Type (+, — or Non-mater) in Progenies of Danish Baking Yeast grown from Asci with the Following Origins:

- Expts. 1-5. Parent stock and four successive generations of inbreeding (F₁ to F₄).
 Expts. 6-8. Three single ascospore cultures showing no mating reaction.
 Expts. 9-11. Single ascospore cultures showing a mating reaction and sporulating capacity, 9 being weak +, 10 strong +, and 11 strong —.
 Expt. 12. Culture grown from a zygote isolated from a — non-sporulating single ascospore culture.
 Expt. 13. Stimulated cells, from a — single ascospore culture, that turned into asci.

No. of spores germinated from ascus	Characters of single ascospore cultures from each ascus	Numbers of asci analysed in expts. 1-13.												
		1	2	3	4	5	6	7	8	9	10	11	12	13
4	2+, 2—	1	—	1	—	—	—	—	—	—	—	—	1	—
	2+, 1—, 1 non-mater	—	—	—	—	—	—	—	—	—	—	—	—	—
	1+, 2—, 1 non-mater	—	1	2	1	1	—	—	—	—	—	—	—	—
	2+, 2 non-mater	—	—	—	—	—	—	—	—	—	—	—	—	—
	2—, 2 non-mater	—	1	—	—	—	—	—	—	—	—	—	—	1
	1+, 1—, 2 non-mater	—	—	—	—	—	—	—	—	—	—	—	—	—
	1+, 3 non-mater	—	—	—	—	—	—	—	—	—	—	—	—	—
	1—, 3 non-mater	—	1	—	—	—	—	—	—	—	—	—	—	—
3	4 non-mater	—	—	—	—	—	—	1	—	—	—	—	—	—
	2+, 1—	—	1	—	—	—	—	1	—	—	—	—	—	2
	1+, 2—	1	2	1	1	—	—	1	—	—	—	2	1	1
	2+, 1 non-mater	—	—	1	—	—	—	—	—	—	—	—	—	1
	2—, 1 non-mater	—	4	4	1	2	—	—	—	—	—	—	—	—
	1+, 1—, 1 non-mater	—	1	—	2	1	—	—	—	—	—	1	—	1
	1+, 2 non-mater	—	—	—	—	—	—	—	—	—	—	—	—	—
	1—, 2 non-mater	—	2	1	2	1	—	—	—	—	—	—	—	—
2	3 non-mater	—	1	—	—	—	—	—	—	—	—	—	—	—
	2+	—	—	—	—	—	—	—	—	—	—	—	1	1
	2—	—	1	3	1	1	—	—	1	—	—	3	—	2
	1+, 1—	—	3	5	2	1	—	—	1	—	—	—	—	2
	1+, 1 non-mater	—	1	—	1	—	—	—	—	—	—	—	1	2
	1—, 1 non-mater	—	3	2	3	1	—	—	—	1	—	—	—	2
1	2 non-mater	—	—	—	—	—	1	1	—	1	—	—	—	—
	1+	—	—	—	—	—	1	1	—	3	2	3	3	2
	1—	—	—	—	—	2	1	2	—	4	1	4	4	1
	1 non-mater	2	—	—	—	—	2	2	—	—	1	—	1	1

The material was next inbred for four generations. The data are given in Table I, expts. 2 to 5. The single-spore cultures in the various generations when analysed for mating reaction and sporulating capacity fell into three classes:

1. Maters, which gave a strong + or — mating reaction with the tester strains. The majority of these did not sporulate at all.

2. Weak maters, only a few cells of which (1 per cent. or less) gave a + or — reaction. The majority of these cultures could sporulate.
3. Non-maters, which gave no mating reaction at all. Most of these cultures could sporulate.

There is a clear negative correlation (Table II) between the presence of a mating reaction and of a sporulation capacity. The 70 asci recorded gave 183 viable progenies out of 280 expected, a viability of 65 per cent. It is unfortunate that all four ascospores from an ascus gave viable cultures in only a few cases (9 out of 70 asci). However, in no case were more than two + cultures or more than two — cultures obtained from one ascus. The cultures which showed no mating reaction replaced one or more of the expected + and — cultures. Amongst the 183 progenies arising from single spores 35 were +, 94 were —, and 54 were non-maters, suggesting that non-maters more readily replace + than — cultures. Indeed, among 7 asci in which all four spores germinated there were 10 non-mater cultures, 9 of which replaced + cultures and only 1 replaced a — culture. Amongst 12 of the three-spored asci there were 11 non-maters replacing + cultures and only 1 replacing a — culture. Assuming this ratio of 10 non-maters derived from + cultures to every non-mater derived from a — culture holds for the group of 54 non-maters, we may assign 5 to — and 49 to +, thus obtaining a ratio of 84 + to 99 — cultures (actually or potentially), which is not significantly different from equality.

TABLE II

The Negative Relationship between Presence of Mating Reaction and Sporulation Capacity in Single-ascospore Cultures of Danish Baking Yeast

Generation.	No. of single spore cultures.	Maters		Weak maters		Non-maters	
		Sporers.	Non-sporers.	Sporers.	Non-sporers.	Sporers.	Non-sporers.
Parent	9	0	7	0	0	2	0
F ₁	61	7	23	6	3	18	4
F ₂	53	6	29	4	3	10	1
F ₃	36	4	16	2	0	10	4
F ₄	24	0	17	0	0	7	0
Total	183	17	92	12	6	47	9
Percentage of spores		15.6		66.6		83.9	

(b) *Behaviour of non-maters.* Besides the usual + and — segregates, Lindgren and Lindgren also found some which gave no mating reaction. This lack of sexual response they presumed to be due not to a lack of mating differentiation but to sterility factors. However, most non-maters in the present experiments would sporulate and so asci from three non-mater single-spore cultures were dissected. As expts. 6 to 8 (Table I) show, + and —

progenies along with non-mating ones were recovered from them in proportions similar to those from crosses between + and — cultures. This shows that the inability of these non-maters to mate is due to the presence of both + and — factors in them and not to sterility factors. Some of the non-mating non-sporers could owe their origin to sterility factors and their absence in F_4 (Table II), when they may have been eliminated by selection, does agree with such a hypothesis, although the numbers are too small to give strong support.

(c) *Sporulation in cultures giving a mating reaction.* To explain the sporulation of single-spore cultures which also gave the mating reaction, Lindegren and Lindegren assumed either illegitimate mating (1943 *a, b, d*) or parthenogenesis (1943 *c*). Asci formed by two + sporulating cultures and one — sporulating culture were analysed with results summarized in Table I, expts. 9 to 11. The + stocks gave — single ascospore progenies in addition to + ones, and the — stock gave + progenies besides — ones. Hence sporulation of these single ascospore cultures which gave the mating reaction is not due to either illegitimate mating or to parthenogenesis.

The situation appeared most puzzling since there is an apparent segregation of mating-type genes at ascospore formation in asci formed by presumed haploids. It was, however, clarified by observing the germination of single spores.

(d) *Observation of spore germination.* On germination each spore buds off cells in succession. These in turn bud off further cells and so on to make a colony of numerous round cells. No sign of conjugation between cells was seen in the great majority of the developing colonies, which were studied until they contained several thousand cells. In a few colonies, however, conjugations were seen to occur between a few of the cells. In several of these cases both zygotes and stimulated cells with conjugation tubes, similar to those seen in deliberate + by — matings, were found.

When zygotes and vegetative cells from two particular single-spore cultures (laboratory stock numbers 52 D 14 *d* and 52 D 1 *a*) were isolated, the cultures from the zygotes consisted of large cells whereas those from the vegetative cells were small celled. Further, cultures from zygotes gave no mating reaction but formed spores, whereas cultures from vegetative cells gave a mating reaction but did not sporulate, at least in the case of those from 52 D 14 *d* (Table III).

To explain the increase in cell size, appearance of sporulation, and loss of mating reaction in cultures from zygotes as compared with cultures from ordinary vegetative cells, two general possibilities may be considered. Either illegitimate mating has occurred, in which case the loss of the mating reaction is difficult to understand, or a mutation of one mating-type gene to the other has occurred followed by conjugation of cells of opposite mating type to form zygotes.

To test whether the zygotes were formed from — $\times$ — matings, since both 52 D 14 *d* and 52 D 1 *a* were —, or — $\times$ + matings, 22 four-spored asci were

dissected from 52 D 14 *d* zygote 2. Germination was very poor, but sufficient single-spore progenies were raised and tested for mating reaction with the results given in Table I, expt. 12. Both + and — single-spore progenies were recovered not only from the stock as a whole but even from individual asci. In one case the four ascospores in one ascus gave two — and two + cultures, but in no case was there more than two of each type per ascus. Since 52 D 14 *d* was —, a mutation to + in at least one of its cells must have taken place. Presumably fusion then followed between + and — cells giving zygotes. These would then have no mating reaction and being legitimate diploids could sporulate and segregate + and — progeny.

TABLE III

Characters of Cultures grown from Zygotes and Vegetative Cells isolated from Single-ascospore Cultures which have little or no Capacity to spore

Designation of single-spore stock	Nature of isolate	Characters of vegetative progeny raised from the isolate		
		Cell size and shape	Mating reaction	Percentage of sporulating cells
52 D 14 <i>d</i>	Zygote 1	Large, round or slightly elliptical cells	None	8 Asci 2- or 3-spored only
	Zygote 2	Large, round or slightly elliptical cells	None	4 Few asci 4-spored
	Three vegetative cells	Each gave small, round or slightly elliptical cells	All	None in any
52 D 1 <i>a</i>	Zygote	Large elliptical cells	None	0.1
	Vegetative cell	Small, round or slightly elliptical cells	—	1
	Vegetative cell	Small, round or slightly elliptical cells	—	4

Additional evidence for mutation for the mating-type genes was obtained by isolating five stimulated cells, six vegetative cells, and two apparent zygotes from a single ascospore culture, 65 1 *c*. The cells in all cultures grown from them were small and round or slightly elliptical, and none of the cultures possessed the capacity to sporulate. One vegetative cell and four stimulated cells proved to be +, while five vegetative cells and one stimulated cell were —. Since the original culture was —, the + cells in the culture must have arisen by mutation of one or more — cells to +.

Unlike the cultures grown from zygotes isolated from 52 D 14 *d* and 52 D 1 *a*, the cultures from the two zygotes isolated from 65 1 *c* showed no increase in cell size, the cells being round or slightly elliptical. The cultures

both showed a + mating reaction and neither culture would sporulate. The significance of these zygotes will not be discussed fully here, but it is possible that the mating producing these zygotes was defective or that cells of the same mating type can conjugate after stimulation, such matings not being followed by sporulation.

It now seems probable that cultures showing both a mating reaction and an ability to sporulate are mixtures of two types of cells, viz. the original haploids together with legitimate diploids derived through mutation and subsequent fusion of cells of opposite mating type. If this were so, it would be the haploids which give the mating reaction and the neutral diploids the sporulation in the culture.

Fourteen cultures were grown from single cells isolated from a — culture, some of whose cells would sporulate. Nine of the cultures gave no mating reaction and a majority (37–68 per cent.) of the cells in each could sporulate, showing thereby their diploid nature. The other five cultures gave a — mating reaction showing their haploid nature, and they either failed to sporulate or did so only in a minority of the cells (14–23 per cent.). Similar results were obtained with a + sporulating culture, 52 D 3 *b*.

The mixed nature of the cultures capable of mating and sporulating was thus revealed. However, since most of the cultures with a mating reaction could also sporulate to some extent it was still doubtful whether sporulation in these cases was due to subsequent mutation and diploidization or whether some of those cells which could give a mating response would also sporulate. If the former were true, a negative correlation between the proportion of cells giving a mating reaction and the proportion of cells sporulating might be expected.

Twelve cultures were grown from single — cells which had been stimulated to produce conjugation tubes by mixing with a few + cells. Each culture showed a mating reaction and also some sporulation capacity, but there was no evidence of any correlation between mating and sporulating capacities (Fig. 1, open circles), except that those showing no mating reaction consistently had a much higher sporulation index. The same behaviour was shown by samples from two other sub-cultures from the same stock (Fig. 1, dots and triangles).

An attempt was made to test both the mating and sporulating reactions in the same cell. A — sporulating stock was mixed with a + non-sporulating tester strain and after 24 hours, when the cells had been stimulated, the culture was put to sporulate. It was examined for sporulation after 5 days, the cells in a sample being classified also for presence or absence of conjugation tubes. It was expected that, if mating and sporulating capacities did not occur together, all cells with conjugation tubes in the culture should not have spored. However, a few such cells did sporulate (Fig. 2), the frequency being much lower than amongst cells with conjugation tubes, namely 5 in 171 (2.8 per cent.) as compared with 1,153 among 3,185 (36.2 per cent.) round cells in the same sample.

Some of these asci which had been formed apparently by stimulated cells were dissected and the single spore cultures were tested for mating type reaction (Table I, expt. 15). The recovery of + and — spores from the same ascus shows the parent cell was a zygote. Possibly the sporulating cell was

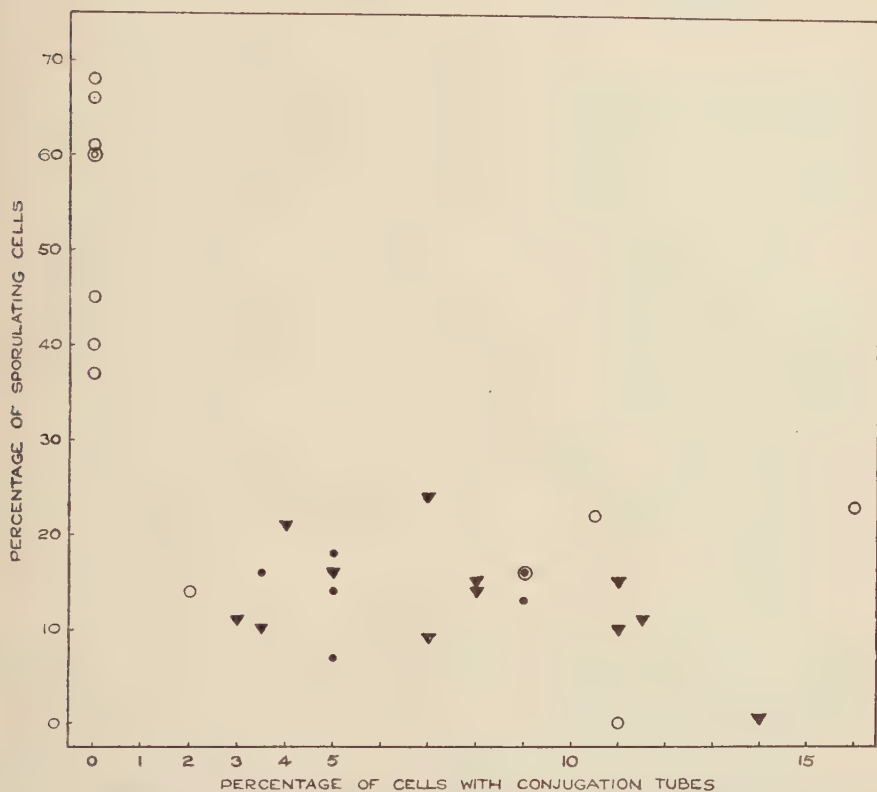


FIG. 1. Relation in a series of cultures of the indices of mating capacity and sporulation capacity, expressed as percentages of cells showing the respective characters. The circles, dots, and triangles represent different subcultures from the same stock.



FIG. 2. Asci apparently formed from cells with conjugation tubes. ($\times 700$.)

of abnormal shape simulating a cell with a conjugation tube. Possibly, even, the zygote may have come from mutation to + of one of the two daughter nuclei in a dividing cell and subsequent fusion of the + with the — nucleus, without completion of cell division. Sporulation could not have involved haploid meiosis, parthenogenesis, or fusion of nuclei of the same mating type. Polyploidy is unlikely because never more than two + or two — single spore

cultures have been isolated from the same ascus. Also the stock (52 D 1 d) from which these asci come was not a pure culture but comprised at least two kinds of cells, namely, non-mating cells which sporulate (diploids) and mating cells whose descendants may or may not sporulate. The latter will produce the former, but not vice versa.

II. MATING SYSTEM IN OTHER STRAINS OF *SACCHAROMYCES*

Three other yeasts have been tested with regard to their mating systems:

Saccharomyces sp. Form 237 (Professor Winge, Copenhagen) proved to be heterothallic with the same general situation as in Danish Baking Yeast. Single-spore cultures are either + or — or non-maters. Maters usually did not spore while non-maters did.

Saccharomyces cerevisiae, Brewers' Yeast (Professor Hopkins, Birmingham). Single-spore cultures in this strain diploidized very early. The four spores from an ascus gave no mating reaction either with the tester strains from the Danish Baking Yeast or when mated together in all possible combinations. All the single spore cultures were non-maters and also sporulated. This strain of *S. cerevisiae* appears to be homothallic.

Saccharomyces carlsbergensis (Delft, Holland) formed two-spored asci. Single-spore cultures were + or — or non-mater. It thus proved to be heterothallic, with a mating system similar to that of Danish Baking Yeast.

DISCUSSION

Winge and Laustsen (1935-44) showed that *Saccharomyces* reproduces sexually with a regular alternation of haploid and diploid phases. The ascospores germinate to produce round and haploid cells which fuse to give elliptical and diploid cells which then multiply vegetatively. Diploidization may also occur by fusion of ascospores. The diploid cells, under suitable cultural conditions, turn into asci, each usually containing four ascospores. No evidence of a parthenogenetic organization of spores was found by these workers.

They found that single-spore cultures would diploidize and sporulate normally, and concluded that these yeasts were homothallic, remarking, 'In these yeasts the fertilization does not consist in a union of cells that differ genotypically, nor do the cells have to differ phenotypically. It is not a question of "+" or "-" cells. . .'

Lindegren and Lindegren (1943 *a, b, c*, and *d*) demonstrated that several yeasts were heterothallic, two of the four ascospores in an ascus belonging to one mating type and the other two ascospores to a different mating type. Mating normally occurred only between cells of opposite mating type. However, it was agreed (with Winge and Laustsen) that mating could occur exceptionally between cells descended from a single ascospore and hence presumably of one genotype. Such matings, however, gave inferior diploid strains with small, more or less round cells, slower growth rate, and poor sporulation capacity. Spores were formed by only a few cells, four-spored

asci were either rare or absent, and the viability of the spores was low. These homozygous matings were designated as illegitimate, the normal $+$ $\times$ $-$ matings being called legitimate. Sporulation in single-spore cultures was said to follow from illegitimate diploidizations or occasionally from parthenogenesis. Besides the normal $+$ and $-$ segregates, the Lindegrens found some which gave no mating reaction. This was ascribed to sterility factors.

The analysis of Danish Baking Yeast presented above shows it to be heterothallic. A majority of the ascospores gave progenies which were either $+$ or $-$ and usually did not sporulate. Since not more than two $+$ or two $-$ spores were isolated from any four-spored ascus, heterothallism in this yeast, as in others, is determined by two allelic genes. This agrees with the Lindegrens' conclusions.

A number of single-spore cultures isolated from it, however, had no mating reaction and, in contrast to the cultures showing a mating reaction, usually sporulated. Such cultures suggest homothallism, the interpretation to which Winge was led. However, the appearance of homothallism is spurious, for the ascospores produced in the asci of such stocks germinate to produce $+$ and $-$ cultures. Nor is there any evidence of genetic sterility or of parthenogenesis. These diploids arising in cultures from single ascospores must therefore have arisen by fusions between $+$ and $-$ haploid cells or nuclei. Each ascospore is presumably originally $+$ or $-$ and the appearance of, for example, $-$ cells in a $+$ culture could only arise by mutation of mating-type genes. Direct evidence of this was obtained by microscopic observation of the growth of colonies from single ascospores. In a few colonies, cells with conjugation tubes and zygotes formed by cell fusion were observed after a considerable number of cells had been formed. Cultures grown from such zygotes had no mating reaction and could sporulate, while those grown from vegetative cells of the same culture possessed a mating reaction (either all $+$ or all $-$) and did not sporulate. Some cultures grown from cells with conjugation tubes, isolated from a $-$ culture, were $-$ and others $+$. The latter must have arisen through mutation of $-$ to $+$, or have descended from a cell in which this had occurred. These observations serve to reconcile the conflicting opinions of Winge and Lindegren. When cells of opposite mating type appear in a single-spore culture as a consequence of mutation, cells are stimulated to produce conjugation tubes. Conjugation and zygote formation usually occurs between cells of opposite mating type. If fusion of cells of the same mating type should occur the resulting progeny of small round cells would give a mating reaction but not sporulate. Proof of the occurrence of this behaviour is difficult to secure.

Following mutation of a mating-type gene the culture becomes a mixture of haploid and diploid cells, haploids giving the mating reaction and diploids the sporulation. The haploid and diploid cells appear to multiply at differential rates with the result that the haploid cells become scarce and finally disappear in subcultures. The growth rate of diploids has been found to be

higher than that of haploids, as both Winge (1935) and Lindegren (1945 b) have previously reported.

Nearly all of the observations described in this paper are accounted for in this way. However, in one experiment (Table I, expt. 15) ascospores were

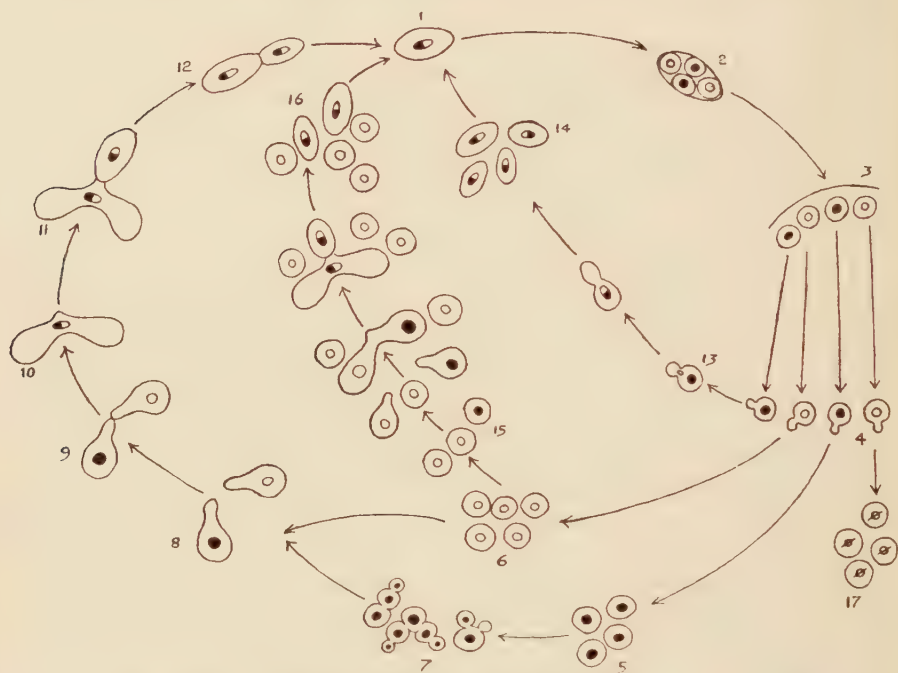


FIG. 3. The life-cycle of heterothallic *Saccharomyces cerevisiae*. 1. A normal diploid cell. 2. Formation of an ascus by a diploid cell. 3. Four haploid spores from the ascus. 4. Spores germinating by budding. 5, 6. Small, round, haploid vegetative cells. 7. Budding of haploid cells. 8. Formation of gametes with conjugation tubes. 9. Fusion of + and - gametes. 10. Formation of zygote and fusion of + and - nuclei to give a diploid nucleus. 11. Budding of large elliptical diploid cells from zygote. 12. Diploid cells budding. 13. Mutation to the opposite mating type of one of the two daughter nuclei of a budding cell. 14. Diploid cells formed by fusion of mutated nucleus with original nucleus in germinating spore. 15. Mutation to + mating type of a cell in a haploid single-spore culture. 16. Formation of diploid cells in a haploid single-spore culture as a result of fusion of a + cell, arisen by mutation, with a - cell. 17. Sterile cells, a source of *Torula*. The black nuclei represent + and the white nuclei - mating type. The diploid nuclei, resulting from the fusion of + and - nuclei, have been shaded half black and half white.

formed in 5 out of 171 cells which showed conjugation tubes but no evidence of conjugation with other cells to form zygotes. The culture in which this occurred was - mating type, but ascospores isolated from these cells with conjugation tubes gave + as well as - cultures, never more than two + or two - spores being isolated from any four-spored ascus. The segregation of opposite mating types in these asci must also come from a mutation to the opposite mating type. The simplest explanation, assuming that we are not

in fact dealing with normal zygotes of abnormal shape, is that the stimulated cells are binucleate and that one of the nuclei has mutated.

The various paths to diploidy and the behaviour of the mating-type genes in the life-cycle of yeast are shown diagrammatically in Fig. 3.

Finally it may be stated that while mutation of the mating-type alleles gives a false appearance of homothallism in the heterothallic strains of *Saccharomyces*, some *Saccharomyces* species are definitely homothallic. Such is the case with a brewers' yeast investigated by the author. Ephrussi, also, has reported a homothallic strain of *Saccharomyces cerevisiae* (Lindegren and Lindgren, 1946).

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Bacterial Symbiosis in Some New Zealand Plants

BY

GRETA B. STEVENSON

With Plate XIX

ABSTRACT

A large number of species of *Coprosma* (Rubiaceae) show bacteria-containing stipular nodules. Hairs which contain bacteria are also present. In other species, for example *Myoporum laetum*, bacteria are present in superficial and in immersed leaf-glands. It is suggested that the bacterial symbionts, which can readily be obtained in culture, may function in the fixation of atmospheric nitrogen and thereby be of importance to the plants when growing in nitrogen-deficient soils.

IT is claimed by a number of workers, for example von Faber (1912, 1914), Boodle (1923), and Humm (1944), that some woody plants of the family Rubiaceae have nitrogen-fixing bacteria associated with the leaves in which bacterial nodules are formed. In New Zealand this family is represented by a number of herbaceous forms and by the woody genus *Coprosma* with nearly forty species, ranging from small trees with large leaves to small-leaved spreading or creeping shrubs. On all the large-leaved species, bacterial nodules are conspicuous on the stipules: *C. arborea*, *C. australis* (Pl. XIX, Fig. 1), *C. lucida*, *C. robusta*, *C. tenuifolia*, and *C. cathartica* with a single apical nodule, up to 5 mm. long (*C. lucida* occasionally with 2 or 3); *C. macrocarpa*, *C. repens* (Pl. XIX, Fig. 2), and *C. serrulata* with several smaller nodules on each stipule. The following smaller-leaved species have one or more slender less conspicuous, stipular nodules: *C. acerosa*, *C. areolata*, *C. banksii*, *C. ciliata*, *C. colensoi*, *C. crassifolia*, *C. cuneata*, *C. foetidissima*, *C. linariifolia*, *C. microcarpa*, *C. polymorpha*, *C. propinqua*, *C. pseudocuneata*, *C. retusa*, *C. rhamnoides*, *C. rotundifolia*, *C. rugosa*, and *C. virescens*. Definite nodules have not been seen on the stipules of the remaining species.

Examination of sections of fresh or fixed material of several species has shown that the larger stipular nodules contain a well-developed palisade-like layer of cells containing masses of bacteria (Pl. XIX, Figs. 1 and 3). The smaller nodules are less regular, consisting of an aggregation of elongated bacteria-containing cells.

Some stipular nodules on the species listed above are accompanied by hairs, especially in those species which have hairs on other parts of the plant. Throughout the whole genus, domatia are present on the under-surface of the leaves, and these pits are ringed round or partly closed with hairs similar to those found on the stipules or elsewhere on the plant. The young hairs of the

domatia and those on the stipules and other parts are densely filled with bacteria. The young buds are also covered with a gum containing bacteria.

In all cases a red pigment was seen to be present at some stage either in the cells which contain bacteria or in neighbouring ones, an observation which may be of interest in view of the importance of red pigments in the N-fixing processes of leguminous nodules. Whether or not the bacterial associates of *Coprosma* fix nitrogen has not been investigated; but many species, particularly the large-leaved *C. australis*, *C. repens*, and *C. robusta*, are common plants that make vigorous growth in gravel or in stony ground which is highly deficient in nitrogenous compounds.

Calcium oxalate crystals are present in some leaf cells and are especially noticeable in cells of the young stipule beneath the bacterial tissue. This stipular calcium oxalate largely disappears as the bacterial tissue ages and withers, suggesting that the oxalic acid may be used up in processes connected with the activity of the bacteria. If these are N-fixing bacteria as in other Rubiaceae plants, the oxalic acid may be the starting-point for the synthesis of amino acids. In general calcium oxalate crystals in plants may not be 'waste products' but may function in some such manner in connexion with nitrogen metabolism.

Bacterial leaf nodules which have been described in Rubiaceae and other plants appear to be enlarged leaf glands. Although New Zealand Rubiaceae do not have leaf glands, there are other New Zealand plants which do have conspicuous leaf glands and which have been found to contain bacteria: *Myoporum laetum* (Myoporaceae), *Metrosideros tomentosa*, *Leptospermum scoparium*, and *L. ericoides* (Myrtaceae) are examples. Buds of *Myoporum laetum* are covered with a reddish gum containing bacteria, and glands full of bacteria are present on the surface of bud leaves as well as within them (Pl. XIX, Figs. 4 and 5). The opened young leaves are green, with colourless bacterial glands immersed in the green tissue (Pl. XIX, Fig. 6). Bud leaves of *Metrosideros tomentosa*, *Leptospermum ericoides*, and *L. scoparium* have similar smaller glands in the leaves, which characteristically show red pigments as well as green; these species also have hairs on the leaves and twigs, and in the buds the young hair-cells contain bacteria. Two Australian species of *Eucalyptus* growing in cultivation have been examined and the glands of the bud leaves were also seen to contain bacteria; red pigments were noticeable in these buds.

Isolations have been made from the stipular glands of *C. australis*, *C. foetidissima*, *C. lucida*, *C. repens*, and *C. robusta*, from the leaf glands of *Myoporum laetum*, and from the two species of *Eucalyptus*. In each case a bacterium which appeared to be the infective organism was isolated on a leaf extract—glucose medium. The properties of the isolates in cultivation have not been examined, and whether or not bacteria-free plants could be raised or reinoculated is unknown.

The bud leaves of many plants have glands similar to those which have been examined in some detail as recorded above. Many which the writer has

examined cursorily appear also to contain bacteria, e.g. *Vitex lucens* (Verbenaceae), *Nothopanax* spp. (Araliaceae), *Suttonia* spp. (Myrsinaceae). It is therefore suggested that bacterial symbionts of higher plants may prove to be more common than has been generally supposed. In the *Coprosma* spp. the symbiont is possibly an N-fixing bacterium like that found in other Rubiaceous plants, but it has been pointed out by Humm (1944) that the bacterial symbionts found in leaves may supply compounds of the nature of hormones or growth substances to the partner plant. However, the question of the nitrogenous nutrition of plants growing in many natural habitats is puzzling, for although plants will readily absorb nitrates and ammonium compounds in culture, such substances are not found in many types of natural soil, particularly in forests, where many species make vigorous growth. If absorption of atmospheric nitrogen takes place through fungi in mycorrhiza as appears likely in many cases, or through bacteria in leaf and stem structures, the plants would be independent of soil nitrogen. Experimental work on N-fixation by green leaves has not yet produced convincing evidence that this occurs, but if experiments were repeated with bud leaves provided with glands, instead of with mature green leaves, results might be different. A wide field for botanical investigation is thus indicated with regard to bacterial symbiosis in glands and glandular hairs of young shoots, and the possible function of the bacterial symbionts.

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EXPLANATION OF PLATE XIX

Illustrating Greta B. Stevenson's article on 'Bacterial Symbiosis in some New Zealand Plants'.

FIG. 1. Longitudinal section of stipular nodule of *Coprosma australis* showing bacterial tissue. ($\times 56$.)

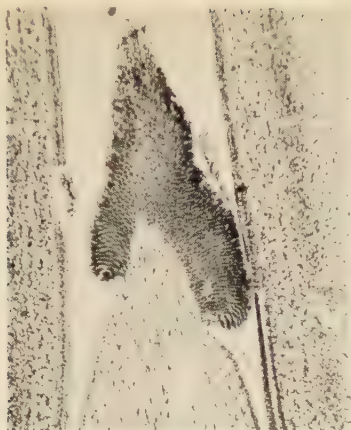
FIG. 2. *C. repens* shoot with several small nodules on stipule. ($\times 7$.)

FIG. 3. Longitudinal section of bud of *C. repens* showing stipular glands with palisade-like bacterial layer. Apparently empty cells in stipular tissue contained calcium oxalate crystals. ($\times 70$.)

FIG. 4. Longitudinal section of bud of *Myoporum laetum* showing glands on leaf surface. ($\times 70$.)

FIG. 5. Transverse section of leaf of *M. laetum* with immersed bacterial glands. ($\times 60$.)

FIG. 6. Portion of *M. laetum* leaf showing bacterial glands. ($\times 7$.)



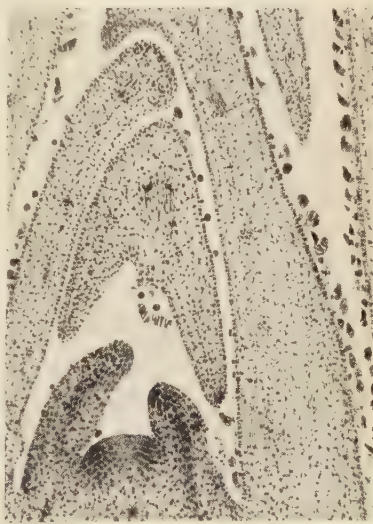
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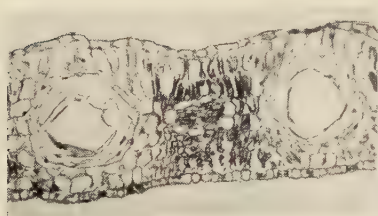
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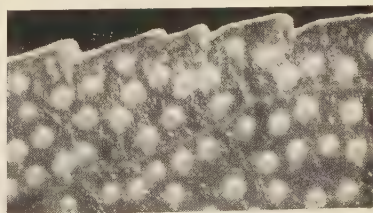
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6

G. B. STEVENSON

Addenda Clavariaceae. III

BY

E. J. H. CORNER

(Botany School, Cambridge)

With four Figures in the Text

ABSTRACT

1. The following novelties are described, with notes on other species of their genera:

Aphelaria subgen. *Tremellodendropsis* for the *A. tuberosa* alliance with sub-tremellaceous basidia: *A. amboinensis* (Lév.) comb. nov.

Chaetothyphula gelatinosa sp. nov. and *C. tetraspora* sp. nov. from Tropical America, whence *C. hyalina* (Jungh.) Corner is also recorded.

Clavariadelphus junceus (Fr.) Corner is recorded from Brazil.

Mucronella flava sp. nov. from Iowa.

Phaeoaphelaria australiensis gen. et sp. nov. is intermediate between *Aphelaria* and stereoid fungi.

Pistillaria trispora sp. nov. from Iowa: *P. tucumanensis* (Speg.) comb. nov. for *Typhula tucumanensis* Speg.

Pistillina calyx Heim is excluded as marasmioid.

2. The *Physalacria*-subseries is reviewed, and the following are described as new:

Hormomitaria albidula sp. nov. from Brazil.

Pseudothyphula gen. nov. from West Africa: *P. ochracea* sp. nov. as the type-species: *P. tenuipes* (Lloyd) comb. nov. (= *Mucronella tenuipes* Lloyd).

APHELARIA Corner

AFTER studying further collections of this genus, I have decided that the species with clamped hyphae and subtremellaceous basidia, such as *A. tuberosa*, must be separated as a distinct group from those with normal basidia and without clamps. As the species are few and, as yet, little known, I refer them to a new subgenus *Tremellodendropsis*. To the naked eye the species appear practically indistinguishable, unless *A. tuberosa* is the only one that tends to a stereoid form when young: the fruit-bodies of the others resemble exactly the forms of *A. dendroides* which are illustrated in my monograph (Figs. 53-55), and they also dry dull brown, horny and twisted, so as to suggest *Pterula*. The four species of subgen. *Tremellodendropsis* differ mainly in spore-size, to a lesser extent in spore-form, the longer spores of *A. tuberosa* being more pointed than the shorter, obtuse or clavate, spores of the others. Further collections may show that it is necessary to unite some of them.

Concerning the distribution of the subgenus there can at present be only an indication, thus:

<i>A. tuberosa</i>	.	.	.	north temperate, south Brazil.
<i>A. flagelliformis</i>	.	.	.	New Zealand, Sumatra, Brazil.
<i>A. pusio</i>	.	.	.	New Zealand, Australia, Madagascar, Brazil.
<i>A. tasmanica</i>	.	.	.	New Zealand, Tasmania.

A second aberrant group is represented by *A. amboinensis* (Lév.) comb. nov., as described in this paper: unfortunately all the collections yet made have been sterile.

KEY TO TREMELLODENDRON AND APHELARIA

Basidia subglobose, tremellaceous: hyphae without clamps, becoming very thick-walled	.	.	.	.	.	.	.	<i>Tremellodendron</i>
Basidia clavate: hyphal walls slightly thickened, often horny and agglutinated on drying	.	.	.	.	.	.	.	<i>Aphelaria</i>
Without clamps: basidia normal	.	.	.	.	.	.	.	subgen. <i>Aphelaria</i>
With clamps: basidia subcruciate at the apex, with long, stout sterigmata	.	.	.	.	.	.	.	subgen. <i>Tremellodendropsis</i>
Subgen. <i>Aphelaria</i> subgen. nov.								
Hyphis haud fibuligeris: basidiis clavatis, haud ad apicem subseptatis: typus, <i>A. dendroides</i> (Jung.) Corner.								
Subgen. <i>Tremellodendropsis</i> subgen. nov.								
Hyphis fibuligeris: basidiis grandibus, clavatis, ad apicem cruciatim subseptatis: sterigmatibus magnis: typus, <i>A. tuberosa</i> (Grev.) Corner.								

KEY TO THE SPECIES OF SUBGEN. TREMELLODENDROPSIS

Spores broadly ellipsoid

Sp. 8-11 × 5-7 μ	.	.	.	.	.	.	.	<i>A. flagelliformis</i>
Sp. 9-15 × 7-9 μ	.	.	.	.	.	.	.	<i>A. tasmanica</i>

Spores elongate, twice as long as wide

Sp. 9-15 × 4-7 μ, mostly oblong	.	.	.	.	.	.	.	<i>A. pusio</i>
Sp. 14-20 × 5-9 μ, elongate amygdaliform	.	.	.	.	.	.	.	<i>A. tuberosa</i>

A. amboinensis (Lév.) comb. nov.

Basinym: *Thelephora amboinensis* Lév., Ann. Sci. Nat. 3, 2, 1844, 207.

Syn.: *Thelephora funalis* Lév., l.c. 208: *Lachnocladium funale* (Lév.) Sacc. (1888).

Thelephora scoparia Lév., l.c. 207: *Lachnocladium scoparium* (Lév.) Sacc. (1888).

—6.5 cm. high, 4 cm. wide, branched, fastigiate, drying light cinnamon fawn to fuscous fawn: stem —2.5 cm. × 3-5 mm., evidently deeply rooting, somewhat fibrillose tomentose: branches multifid below and flattened 2-5 mm. wide at the axils, dichotomous above and 1-2.5 mm. wide, with acute tapering subulate branchlets 0.5-1 mm. wide, the internodes rather elongate, drying twisted and glabrous: hymenium?: flesh fibrillose on drying, not horny.

On the ground: Java, Amboina, Aru Isl.

Hyphae $3-7\mu$ wide, monomitic without clamps, the branches constricted at their origin, the walls light brownish and more or less strongly thickened -2.5μ , not inflating, longitudinal and entangled.

Gloeocystidial branches of the peripheral hyphae $-150 \times 6-10\mu$, colourless, thin-walled, subcylindric, evidently with oleaginous contents when alive, longitudinal and immersed in the superficial tissue, with blunt tips, mainly on the younger branches.

Collections: types of *T. amboinensis*, *T. funalis*, and *T. scoparia* in the Kew herbarium: Challenger Exped., Aru Isl, Sept. 1874, in the Kew herbarium (ut *Lachnocladium furcellatum*): Zollinger, Planta Javanica 1445 D, Prabakti, in Herb. Lugd. Bat. n. 910.222-3476.

There is no trace of the hymenium on any of these five collections. The wide hyphae, fibrillose texture of the dried fruit-bodies, and gloeocystidial branches distinguish them from *A. dendroides*, to which I tentatively referred the species of L  veill   in my monograph.

Compare *A. trachodes* from Brazil.

A. aurantiaca (P. Henn.) Corner

Spruce s.n., Brazil (Panur   and Mt. Cocui, on the ground: in Kew herbarium as *Lachnocladium furcellatum*).

-10 cm. high, 7 cm. wide, 'sordide luteus, hymenium album' (Spruce): stem $-20 \times 3-6$ mm., somewhat rugose-fibrous with small, irregular, upward projections, arising from a plexus of fine rhizomorphs, fastigiate divided: branches dichotomous, appearing polychotomous below, not verticillate, $1-2$ mm. wide below, narrower and rather divaricate upward, with filiform tips: drying dull orange drab to light cinnamon drab, with deeper coloured medulla c. 0.5 mm. wide.

Spores $5.5-6.2 \times 5-6\mu$, white, subglobose, thin-walled, the apiculus 0.5μ long.

Basidia $35-45 (-60) \times 7-8\mu$, not septate: sterigmata 2-4.

Hymenium thickening -350μ : no cystidia.

Hyphae $2-4.5\mu$ wide, without clamps, the walls firm or slightly thickened and colourless in the subhymenium and outer part of the medulla, but the medulla composed mainly of hyphae with orange-brownish walls, -1μ thick, darkening in dil. alkali: the medulla extending, as the main part of the flesh, to the tips of the branches and becoming paler distally.

This species is near to *A. tropica* in its spores, but has larger, more branched fruit-bodies of a richer colour and with the distinctive medulla. The type-collection is also represented in the U.S. Dept. Agr. Myc. Collections.

A. dendroides (Jungb.) Corner

Lloyd Cat. No. 32750: Philippine Isl. (det. *Pterula pusio* B.): spores c. $9 \times 8\mu$: hyphae $2-4\mu$ wide, no clamps.

A. flagelliformis (B.) comb. nov.

Basinym: *Clavaria flagelliformis* B., Fl. Antarctica, vol. ii, 1855, 186.

Syn.: *Lachnocladium flagelliforme* (B.) Cke., Handb. Austral. Fungi, 1892, 179.

Pterula squarrosa P. Henn., Hedw. 1902, 6.

—5 cm. high, drying wholly horny-tough and fuscous brown: stem 1–2 mm. wide: branches multifid below and slightly flattened —4 mm. wide at the axils, bifid above.

On the ground: New Zealand (Bay of Islands: type in the Kew herbarium).

Spores 8·5–11 × 6·5–7·5 μ , white, smooth, bluntly ellipsoid to subclavate or lachrymiform, the apiculus 1 μ long; copious.

Basidia? (much collapsed): cystidia none.

Hyphae 2·5–3·5 μ wide, monomitic, not inflated, *clamped*, the walls slightly thickened.

This is the description of the type-collection, which I wrongly referred to *A. dendroides* in my monograph. The hyphae are clamped, and the following collections agree so closely that I cannot distinguish them. Unfortunately the basidia in all are so badly preserved that I could not make out their form. The species seems very close to *A. pusio* with larger spores. The hyphae become much agglutinated in the dried specimens, so that it may seem impossible to make out the presence of clamps: however, on re-examination of the type, I found some tissue near the base of the stem in which the hyphae were not agglutinated, and this enabled me to correct my former identification.

Pterula squarrosa P. Henn., type-collection (on the ground, Pará, Brazil: fragment in U.S. Dept. Agr. Myc. Coll.) and Lloyd Cat. No. 32749 (on dead wood, Bahia, Brazil, leg. C. Torrend): —4 cm. high, rather sparsely branched, fastigiate, the stem arising from a thin whitish mycelium: branches multifid with slightly expanded axils below, dichotomous above: spores 8·5–10 × 5·2–6·5 μ , the apiculus *c.* 1 μ long: hymenium thickening —70 μ : hyphae 2·5–3·5 μ wide, clamped and often branched from the clamp, the walls slightly thickened (0·5 μ).

Lutjeharms, W. J., n. 4496, Sumatra (Enggano Isl., Boeah Boeah, on the ground, June 9, 1936: in Herb. Leiden): —4 cm. high, *pallid white when fresh*, with flattened branching as in *A. dendroides*: spores 10–11 × 5–5·5 μ , blunt: hyphae 3–4 μ wide, clamped, slightly thick-walled.

A. pusio (B.) Corner

Whitelegge s.n., Australia (Double Bay, on dead wood: in herb. Kew, as *Lachnocladium flagelliforme* (B.) Cke.).

—7 cm. high, fastigiate, slender, drying dull ochraceous: *stem* 2 cm. × 2–3 mm., dividing radially into four rather stout, erect, main branches, glabrous, but subtomentose at the abrupt base: *branches* divided 2–4 times, with flattened branching, 2–4-fid below, mainly dichotomous above, with acute

axils and long slender, subparallel tips — $20 \times 0.5-0.8$ mm., not flabelliform, glabrous.

Spores $9.5-15.5 \times 4.7-6 \mu$, white, smooth, thin-walled, subcylindric, blunt, evidently multiguttulate.

Basidia $45-70 \times 12-13 \mu$, as in *A. tuberosa*: sterigmata 4, $10-14 \mu$ long.

Hyphae $2-3 \mu$ wide, clamped, with slightly thickened walls.

Wier n. 14391, Brazil (Parecy, leg. J. Rick, 1918, on the ground): spores $8.5-15 \times 4-6.5 \mu$, oblong: hyphae $2-3 \mu$ wide, clamped.

Whitelegge's collection has larger fruit-bodies than the type of *A. pusio*, but is otherwise identical.

***A. tasmanica* (Lloyd) Corner**

Type-collection, Lloyd Cat. No. 32715, Tasmania (leg. L. Rodway): — 4.5 cm. high, ? caespitose, 3–5 times dichotomous throughout, often with very short internodes below, the branching flattened, drying horny and drab white with the sterile surfaces inclining to rich ochraceous: *stem* — $10 \times 1-1.5$ mm., sterile: *branches* $0.3-0.5$ mm. wide below, terete, tapering to the acute tips, rather lax, the uppersides sterile: *spores* $12-15 \times 7.5-9.5 \mu$, white, smooth, oblong, rounded at the apex, multiguttulate, the apiculus $c. 1 \mu$ long: basidia $60-77 \times 12-13 \mu$, subtremellaceous with the septa extending — 15μ from the apex: sterigmata 4, $10-13 \mu$ long: hymenium thickening — 120μ : hyphae $2.5-4 \mu$ wide, clamped, the walls firm, not inflated.

Warcup n. C 49, New Zealand (in herb. Cantab., with photograph): — 6 cm. high, fasciculate, bushy, with flattened branching, appearing polychotomous below, the axils dilated — 4 mm. wide, dichotomous above, the cylindric branches $c. 1$ mm. wide, branched 4–5 times: *stem* $1-2$ cm. $\times 2-3$ mm., sterile: hymenium absent from the upperside of the main branches: drying fuscous horny with pallid ochraceous stem: *spores* $9.5-12 \times 7-9 \mu$, white, smooth, ovoid, thin-walled, wholly granular-guttulate, the apiculus $1-1.5 \mu$ long: basidia $60-90 \times 11-15 \mu$, subtremellaceous in most cases, but a few basidia apparently not septate, $3-3.5 \mu$ wide at the base, sterigmata 2–3–4, $10-14 \times 3.5-4 \mu$: hymenium thickening, cystidia none: hyphae $2-4 \mu$ wide, clamped, the walls firm, with many secondary septa without clamps.

Warcup's collection has slightly shorter spores than the type, and its hyphae are in places secondarily septate, but I can see no satisfactory means of distinguishing them. The species differs from *A. flagelliformis* in the longer spores.

***A. tropica* (Mont.) Corner**

Corner 786, Brazil (Manaus, Amazonas, on the ground in the forest, June 14, 1947): — 3 cm. high, solitary or subcaespitose, *sparingly branched*, *pallid white* when fresh: stem $3-10$ mm. long, smooth, sterile: *branches* 2–3-chotomous below, dichotomous above, branched 2–4 times, scarcely spreading, slightly flattened at the axils, the tips filiform-subacute then blunt: hymenium amphigenous or only on the lower side of inclined branches: *spores* $7-9 \times$

5–6.5 μ , white, smooth, ovoid or ellipsoid, opalescent, the contents contracting into a gutta-like body in the dried spore, the apiculus 1 μ long: basidia 45–60 $\times$ 10–11, clavate, not septate, with 4 sterigmata 7–9 μ long: hymenium thickening—200 μ no cystidia: hyphae 2–3 μ wide, septate without clamps, monomitic, the walls thickened—0.7 μ , the branches constricted at origin: subhymenial hyphae 3–5 μ wide, thin-walled.

This collection has slightly more ellipsoid spores than described in my monograph. The deep yellow or tawny colour given in the original description refers to the dried specimen. The Asiatic *A. spiculosa*, with slightly larger spores, is very close. *A. aurantiaca* has larger fruit-bodies with a deeper coloured and firmer medulla (at least in the dried specimens) and shorter, subglobose spores.

A. tuberosa (Grev.) Corner

Corner s.n. Brazil (Matto Grosso, Campo Grande, March 23, 1948, on the ground in the forest); —3.5 cm. high, sparsely and dichotomously branched, *pallid alutaceous*, subfuscous below, the tips white, drying brownish and horny: *stem* —12 $\times$ 2 mm.: *branches* 1–1.5 mm. wide, once or twice dichotomous: subcoriaceous, smell sour: *spores* 16–20 $\times$ 7–8.5 μ , white smooth, thin-walled, multiguttulate, the apiculus 1.5 μ long: basidia 55–70 $\times$ 12–15 μ , shortly subcruciate septate, with 4 sterigmata 7–9 μ long, 5–6 μ wide at the base: hyphae 2–5 μ wide, clamped, the walls firm and slightly thickened—0.5 μ , the branches often constricted at their origin.

Except that the fruit-bodies of this collection are slender, regularly dichotomous, and not the least palmate and that the spores are slightly wider, I can see no difference from the north temperate collections. The basidia have no septation at the apex until the sterigmata are nearly full-grown: then, it seems that the bases of the sterigmata suddenly contract together to give a new figure to the dome-like apex of the basidium, whereby it appears to have incipient septation. There are no true septa partitioning the basidium.

CHAETOTYPHULA Corner

C. gelatinosa sp. nov.

Ut *C. hyalina*, sed receptaculis gelatinosis, siccis corneis: cystidiis latioribus, 40–70 $\times$ 9–12 μ , tunica media gelatinosa 2–4 μ crassa, tunica externa tenuissima sed firma: hyphis 2–5 μ latis, tunicis gelatinosis, stipitis superficie inflatis ad 12 μ latis, internis vix inflatis ad 6 μ latis: ad vaginam foliae palmarum, Panama (Prov. Chiriquí, Upper Rio Chiriquí Viejo, alt. 1,800–2,000 m., July 8, 1935, G. W. Martin 2659).

Fruit-bodies as in *C. hyalina* but gelatinous, pallid white, drying horny and yellowish.

Spores 8–9.5 $\times$ 4–5 μ , ellipsoid or cylindric to subpruniform.

Basidia 25–32 $\times$ 7–8 μ : ? 4-spored.

Cystidia 40–70 $\times$ 9–12 μ , abundant, lanceolate, blunt, projecting, the base deeply immersed and often longitudinal, smooth, colourless, thick-walled,

the outer wall very thin but firm, the middle part of the wall much thickened, 4μ , and gelatinous, the obtuse apex thin-walled.

Hyphae monomitic, ? clamped: internal hyphae $2-6\mu$ wide, with gelatinous walls, scarcely inflated, long-celled: the superficial hyphae of the stem $5-12\mu$ wide, with firm, scarcely thickened walls and shorter cells, often with short protuberances (as incipient basidia) to the exterior.

Caulocystidia apparently absent.

The material was scant, but distinctive: it is in Professor Martin's herbarium at Iowa State University.

C. hyalina (Jungh.) Corner

Colombia, G. W. Martin 3678 (Dept. Magdalena, Sierra Nevada de Santa Marta, Hacienda Cincinnati, alt. 1,250-1,500 m., Aug. 24, 1935): on dead pods of *Inga*: spores $7.5-10 \times 4-5.5\mu$: basidia 2-spored: cystidia and caulocystidia typical: hyphae $2-17\mu$ wide, long-celled, without clamps, the walls slightly thickened and agglutinated in the outer part of the stem, not gelatinous.

There is no difference between this rather scant collection and the Malayan fungus. The species is evidently pantropical.

C. tetraspora sp. nov.

Ut *C. hyalina*, sed sporis minoribus, $5.7-7.5 \times 3-3.7\mu$, basidiis tetrasporis, hyphis fibuligeris, cystidiisque minus incrassatis lumine lato praeditis: ad folia emortua in silva, *Brasilia* (Rio de Janeiro, Corcovado, c. 500 m. alt., Nov. 28, 1948, Corner 766, ut typus); *Panama* (Prov. Chiriquí, Upper Rio Chiriquí Viejo, c. 1,700 m. alt., June 30, 1935, G. W. Martin 2214).

—8 mm. high, solitary, gregarious, *white*: stem $2-4 \times 0.15-0.25$ mm., $0.07-0.1$ mm. wide at the apex, tapering upward, pellucid, puberulous: head $4 \times 0.15-0.25$ mm., shortly cylindric, blunt, curving, opaque, rather abrupt.

On dead leaves in the forest: Brazil (Rio de Janeiro, Corner 766, as the type); Panama (Upper Rio Chiriquí Viejo, Martin 2214).

Spores $5.7-7.5 \times 3-3.7\mu$, white, smooth, subcylindric, obtuse, aguttate, thin-walled.

Basidia $17-28 \times 5.7-6.5\mu$, clavate, the young basidia subcylindric: *sterigmata* 4, $3.5-4.5\mu$ long.

Cystidia $25-70 \times 4-8\mu$, frequent or sparse, becoming immersed, cylindric, slightly tapered, or subventricose, the apex blunt or subcapitate, the walls $1-2.5\mu$ thick, *the lumen generally wide*, especially at the apex, not encrusted.

Caulocystidia— $80 \times 2.5-6\mu$, subcylindric and blunt or conical and tapering to an acute, or subacute, often flexuous tip, the walls thin or slightly thickened— 0.5μ , aseptate, scattered or abundant.

Hyphae— 16μ wide, inflating, thin-walled, *clamped*, not encrusted, longitudinal, some not inflated, the cells— 300μ long, $3-5\mu$ wide on the surface of the stem and subagglutinated: in the head, forming a loosely interwoven cortex, $25-35\mu$ thick, of uninflated, divergent, much branched, short-celled hyphae, this layer forming the subhymenium: hymenium not thickening:

many of the hyphae in material preserved in alcohol-formalin having short, acicular crystals in the cell-sap.

This species was exceedingly abundant in places in the forest on the mountain Corcovado, in Rio de Janeiro, during November and December 1948. It differs from *C. hyalina* only in the microscopical details, but these are constant, as shown by Martin's collection from Panama. The following calculations show that the four-spored condition in *C. tetraspora* is not merely a simple multiple of the two-spored condition in *C. hyalina*, for the volume of the basidium and the total spore-volume is different in the two species:

	Mean spore size (μ)	v ($c\mu$)	nv ($c\mu$)	Mean basidium size (μ)	V ($c\mu$)	V/nv
<i>C. tetraspora</i>	6.6×3.35	38.7	155	22.5×6.1	327	2.1
<i>C. hyalina</i>	9.5×5.0	124.0	248	27.0×7.25	557	2.2

Spegazzini's record of *Typhula candida* in Argentina and Bresadola's fungus referred to *T. Traillii*, as mentioned on p. 491 of my monograph, may belong to *C. tetraspora*. The cystidia of this species are often few and become immersed in the thick subhymenium, so that they are easily overlooked: indeed, it is advisable, when studying these tropical species, to examine very young specimens for the projecting cystidia.

CLAVARIADELPHUS Donk

C. fistulosus (Fr.) Corner var. *Orlosii* Pilat

Syn. *C. contorta* Fr. var. *Orlosii* Pilat, Studia Bot. Czechosl., 11, 1950, 153, f. 6.

Habit and size of *C. fistulosus* var. *contortus* but with the smaller spores of *C. fistulosus*: 3 cm. $\times$ 3-4 mm., fasciculate, fuscous ochraceous, hollow, elastic-fleshy: spores $10-15 \times 5-7\mu$: basidia $75-90 \times 9-11\mu$: hyphae -27μ wide: on a broken dead branch of *Betula verrucosa*, hanging on the tree, in virgin forest, Poland (Bialowiecza, leg. A. Pilat, Oct. 14, 1950).

This very interesting find, which Dr. Pilat kindly lent me for study, comes between *C. fistulosus* and the var. *contortus*. In referring var. *Orlosii* to *C. fistulosus*, I have merely followed the nomenclature in my monograph.

C. junceus (Fr.) Corner

Lloyd Cat. No. 30223, leg. J. Rick, Brazil (? São Leopoldo, Rio Grande do Sul): arising from slender, sterile rhizomorphs among dead leaves: spores $7-9.5 \times 4.7-5.7\mu$, aguttate, thin-walled: basidia $c. 28 \times 6\mu$, apparently all 2-spored: caulocystidia feebly developed at the base of the stem: hyphae -18μ wide, cylindric, parallel, clamped, with broad septa, the outer agglutinated hyphae lacking clamps.

This is the first record of the species from South America. The two-spored condition is unusual.

Synonym: ? *Clavaria hortulana* Vel., České houby, 1922, 785, f. 141-5: Pilat, A., Op. Bot. Čech. vi, 1948, 283.

6-12 cm. $\times$ 1-2 mm., cylindric, horny-firm, with long stem-like base, fiery golden-yellow, apex fuscidulous and blunt: spores 8μ , broadly pyriform, greenish yellow: among damp moss and plant-remains in a garden (Czechoslovakia, Mnichovice).

Though said by Velenovsky to be unlike any known fungus, especially in the brilliant colour of the fruit-body and the greenish colour of the spores, yet I think that it is only a state of *C. junceus*. There appears to be no type-specimen.

C. ligula (Fr.) Donk

Herb. Krypt. Musei Nat. Pragae 489921 (South Bohemia, Vodňany, in pinetis muscosis, Aug. 23, 1937, leg. Jos. Herink): —3 cm. high, clavate as diminutive *C. pistillaris*: spores $10-14 \times 3.5-4.5\mu$: basidia $50-60 \times 8-9\mu$.

Weir 15940 (U.S.A., Idaho): spores $13-15 \times 3.5-3.7\mu$.

Weir 16949 (U.S.A., Idaho): basidia $50-70 \times 8\mu$.

C. pistillaris (Fr.) Donk

Herb. Krypt. Musei Nat. Pragae, s.n. (Central Bohemia, Karlstejn, in silva frondosa, Nov. 21, 1946, leg. V. Vacek): spores $10-15 \times 6.5-9\mu$: basidia $11-13\mu$ wide.

Doty 6781 (U.S.A., Harvard, Mass., Nov. 12, 1946): spores $12-15.5 \times 7-8.5\mu$.

This collection of Dr. M. S. Doty shows that the typical, large-spored, form of the species occurs also in North America.

MUCRONELLA Fr.

M. alba Lloyd

Myc. Notes 61, 1919, 880, f. 1509.

—5 mm. long, white, fasciculate, clustered on bark, without a subiculum: U.S.A. (Washington).

Spores $7.5 \times 4.7-5.5\mu$, white, smooth, thin-walled, blunt, the apiculus c. 1μ long.

Basidia $30-40 \times 7-8.5\mu$; sterigmata 4, 6μ long.

Hymenium not thickening: cystidia absent.

Hyphae monomitic, $2-10\mu$ wide, the cells — 150μ long, thin-walled, slightly inflating, clamped, longitudinal, with a few crystals or crystalline masses between the hyphae.

These microscopic details are added from my examination of the type-collection in Lloyd's herbarium. It is a true *Mucronella*, better developed than any other yet discovered, but it sheds no more light on the affinity of the genus.

M. brasiliensis Corner

The following collection from Panama has fruit-bodies identical in size and form with those of *M. pusilla* from Malaya, but there are no crystals among the hyphae which are narrower, more loosely arranged, possibly longer-celled, and more frequently branched from the clamp than in *M. pusilla*. I tentatively refer them to *M. brasiliensis*, but I am not satisfied because I saw occasionally in the subhymenium very narrow, laxly dichotomous hyphae, *c.* 1μ wide, with rigid walls, suggestive of the dichophyses of *Parapterulicium*: their occurrence was very sporadic and I was unable to connect them with the true hyphae of the fruit-body.

Martin, G. W. 2816: — $500 \times 50\text{--}120\mu$, sterile at the apex and base: spores $4.5\text{--}5.5 \times 2.5\text{--}3\mu$, bluntly ellipsoid or shortly oblong: hyphae $1.5\text{--}4\mu$ wide, clamped, often irregularly branched, or branched from the clamp, thin-walled, without crystals: cystidia absent: on dead wood, Panama (Prov. Chirique, Llanos del Volcán, *c.* 1,200 m. alt., July 14, 1935).

M. flava sp. nov.

Usque $2 \times 0.1\text{--}0.2$ mm., conica, acuta, simplex, solitaria vel dense gregaria, ceraceo-lutea; stipite albo-pruinoso, 0.2 mm. longo: sporis $4.3\text{--}5 \times 2.5\text{--}3.2\mu$: basidiis tetrasporis: hyphis $3\text{--}15\mu$ latis, fibuligeris, inflatis, tenue tunicatis, inter se crystallis plus minus tetrahedricis copiosis: ad lignum emortuum, Am. Bor., Iowa, leg. G. W. Martin 6414, Sept. 1950.

—2 mm. high, $0.1\text{--}0.2$ mm. wide at the base, tapering to the acute apex $10\text{--}30\mu$ wide, simple, acerose, downpointing, solitary or densely gregarious, not caespitose, *waxy yellow, with a short white-pruinose stem* —0.2 mm. long.

On dead wood: U.S.A. (Iowa City, leg. G. W. Martin 6414, Sept. 20, 1950).

Spores $4.3\text{--}5 \times 2.5\text{--}3.2\mu$, white, smooth, blunt, shortly oblong ellipsoid, apparently aguttate.

Basidia $14\text{--}17 \times 5\mu$: sterigmata 4, $2.5\text{--}3\mu$ long.

Hymenium not thickening, becoming continuous over the apex of the fruit-body, disorganising from below upward: subhymenium slight, cystidia none: caulocystidia none.

Hyphae $3\text{--}15\mu$ wide, monomitic, clamped, longitudinal *inflating* acropetally from the base of the fruit-body, short-celled near the base, longer celled towards the apex, the cells up to 170μ long, some of the cells sparsely secondarily septate, $2\text{--}2.5\mu$ wide at the growing apex, the walls thin or slightly thickened near the base of the fruit-body.

Crystals, $3\text{--}10\mu$ long, more or less regularly tetrahedral, colourless, very abundant in the tissue, especially on the surface of the stem.

As noted by Professor Martin, who kindly sent me the material for study, this fungus greatly resembles *Hormomitaria sulphurea* from Malaya, but it lacks the characteristic oleocystidia, sterile base to the head, and truly moniliform hyphae. On the other hand, it has the small crystals which seem characteristic of *Mucronella*. That a few of the hyphae should show some degree of secondary septation, at the base of the fruit-body, is interesting because

this feature, when added to the general resemblance with *Hormomitaria*, suggests that *Mucronella* may be connected with the *Physalacria*-series. Indeed, *Hormomitaria albidula*, described in this paper, also lacks the oleocystidia.

PHAEOAPHELARIA gen. nov.

Receptaculis clavarioideis dein stereois, ramulis dichotomis v. multifidis, ligulato-subspathulatis, fastigiatis, dein divergentibus, inferne hymenio amphigeno, superne hymenio unilaterali praeditis: carne fusco-brunnea, subcoriacea, sicca duriuscula: sporis hyalinis, levibus: hymenio incrassato, cystidiis deficientibus; hyphis monomiticis, haud inflatis, haud fibuligeris, tunicis brunneolis, leniter incrassatis: ? lignicola, Australia, specie unica *P. australiensis* ut typo.

***P. australiensis* sp. nov.**

Fig. 1.

—3 cm. alta, ramulis 1.5–2.5 mm. latis, apicem versus spathulato-flabelliformibus 3–6 mm. latis: sporis 4–5 × 3–3.5 μ: basidiis 35–55 × 4–6.5 μ, tetrasporis; hyphis 2.5–6.5 μ latis: ad basim trunci, pr. Sydney, Australia (J. B. Cleland n. 521, Lloyd Cat. No. 33030).

—3 cm. high, clavarioid then stereoid, with numerous branches 1.5–2.5 mm. wide from a short, narrow, stem-like base: branches ascending, flattening, spreading and becoming narrowly spathulate-flabelliform 3–6 mm. wide, dichotomous or multifid but branched only 1–3 times, drying fuscous brown with pallid grayish white hymenium: evidently fibrous-coriaceous, but drying hard and brittle.

At the base of a trunk: Australia, pr. Sydney.

Spores 4–5 × 3–3.5 μ, white, smooth, bluntly ellipsoid, thin-walled, with small apiculus.

Basidia 35–55 × 4–6.5 μ, clavate with narrow base: sterigmata 4, 4.5–6 μ long, straight and stiff, persisting in the collapsed basidia.

Hymenium thickening, present all round the lower branches, but sterile on the uppersides of the oblique flabelliform branches: cystidia absent.

Hyphae 2.5–6.5 μ wide, monomitic, not inflating, without clamps (not even on the basidia), with slightly thickened, pale brown walls (darkening in dil. potash), the subhymenial hyphae colourless: not constricted at the septa.

Old tissues giving a brown colour dissolving in dilute potash.

On the old system of classification the young fruit-bodies of this fungus would be classed with *Clavaria*, the old with *Stereum*. On microscopic examination, however, one soon finds that these form-genera are artificial aggregates,



FIG. 1. *Phaeoaphelaria australiensis* (Lloyd) comb. nov.: fruit-body in section, × 1: basidia and spores, × 1,000 (Cleland 521)

and, just as *Aphelaria* can be separated from other clavarioid genera, so I think certain tropical species of *Stereum* will be found that are allied with *Aphelaria*. *P. australiensis* is such an intermediate.

PISTILLARIA Fr.

P. fuegiana (Speg.) Corner

The type of this species (Inst. Spegazzini 19260) consists of a piece of *Nothofagus*-bark with black spots, on one of which is a fruit-body and the stalk of another. Examination of a small piece of the fruit-body revealed only a mass of very narrow, submucilaginous hyphae, *c.* 1μ wide, without clamps, but there appeared to be young pyriform basidia, $8-9\mu$ wide, embedded and scattered among the hyphae at the surface. I saw no sterigmata or septation of the basidia and no cystidia. There were numerous spores, including many mould-spores, but there were a few typical amygdaliform basidiospores, with lateral apiculus, $8-9 \times 6-7\mu$, and many narrow, cylindric spores $4-6.5 \times 1.5-2\mu$ and $6-7 \times 2-3\mu$, as Spegazzini gave. Elucidation must await rediscovery, which should not be difficult for anyone able to visit Tierra del Fuego.

P. micans Fr.

U.S. Dept. Agr. Myc. Coll. (California, Colton, leg. C.G.S., April 23, 1939): — $1\text{ mm.} \times 150\mu$, subcylindric, flesh colour, fertile from the base: spores $8.5-10.5 \times 6-8\mu$, lachrymiform: basidia $28-35 \times 9.5-10.5\mu$, with 2 stout sterigmata: no cystidia: hyphae $2.5-4\mu$ wide, — 8μ in places, clamped, thin-walled, not agglutinated: on dead *Eucalyptus*-leaf.

P. montevidensis Speg.

I could find no fungus in the type-packet (Inst. Spegazzini n. 19266, leg. Herter n. 396, Toledo, Montevideo, Jan. 13, 1910).

P. trispora sp. nov.

Fig. 2.

1.5 mm. alta, pusilla, solitaria v. gregaria, alba, sicca cornea: stipite $200-300 \times 170-220\mu$, brevi, glabro: capitulo $1\text{ mm.} \times 200-300\mu$, subclavato, subcylindrico v. conico. minute pruinoso: sporis $6-10 \times 4-5\mu$, subcylindricis, obtusis: basidiis $23-40 \times 7.5-9.5\mu$, saepius septo ordinis secundis unico divis, sterigmatibus 2-3, $5-13\mu$ longis: cystidiis deficientibus: hyphis $2.5-6\mu$ latis, hinc inde— 8μ , tunicis subgelatinosis, fibuligeris vel saepius ordine secundo septatis: ad folia emortua *Fraxini*, Am. Bor., Iowa City, leg. G. W. Martin, n. 6359, Oct. 4, 1947.

— 1.5 mm. high, solitary or gregarious, simple, *very small, white*, evidently waxy-subgelatinous when fresh, drying horny: *stem* $200-300 \times 170-220\mu$, *short, glabrous*, cylindric, slightly inserted at the base: *head* $1\text{ mm.} \times 200-300\mu$, subclavate, subcylindric or conical, minutely pruinose.

On dead leaves of *Fraxinus*: U.S.A. (Iowa City, October).

Spores $6-10 \times 4-5 \mu$, white, smooth, thin-walled, ellipsoid-subcylindric, obtuse, the apiculus 0.5μ long.



FIG. 2. *Pistillaria trispora* sp. nov.: fruit-bodies, $\times 5$; basidia, spores, and hyphae, $\times 1,000$ (Martin 6359)

Basidia $23-40 \times 7.5-9.5 \mu$, clavate, mostly without clamps, secondarily septate at maturity with a septum placed $5-15 \mu$ from the apex: sterigmata 2-3, $5-13 \mu$ long, very variable in length even on the same basidium.

Hymenium not thickening: subhymenial hyphae with short cells $5-12 \times 2.5-4 \mu$, compact, apparently without clamps: cystidia none.

Hyphae $2.5-6\mu$ wide, in places -8μ , with thin, subgelatinous walls, *some septa with clamps but most secondarily septate without clamps*, long-celled: on the surface of the stem $1.5-3.5\mu$ wide, with slightly thickened, agglutinated walls: caulocystidia none: construction as in *P. setipes*.

This collection of many small fruit-bodies was referred by Professor Martin to *P. typhuloides* (Notes on Iowa Fungi XI, Iowa Ac. Sci, 55, 1948, 200, f. 1). But the constantly small size of the fruit-bodies, their different habitat, the secondary septation of the basidia and of the hyphae, the uncertain presence of clamps, and the 2-3 sterigmata induce me to describe it as new. Some of the basidia are functionally monosporous through abortion of the one or two other sterigmata. Most of the medullary hyphae are secondarily septate, but some bear rather large clamps, separated widely by septa without clamps.

I examined five fruit-bodies of Martin's collection. All had on the hymenium many long, cylindric or rod-like, thin-walled conidia, $20-30 \times 2-2.5\mu$, with rather truncate ends. I could not find that they were attached, but it seemed that they might have been endoconidia extruded from narrow hyphae among the basidia.

***P. tucumanensis* (Speg.) comb. nov.**

Basynym: *Typhula tucumanensis* Speg., An. Mus. Nac. Buenos Aires, 19, 1909, 280: Corner, Monogr., 1950, 524 (sub *Pterula tenerima* P. Henn.).

$-30 \times 0.2-0.25$ mm., erect, simple, filiform, very acute, (? white): stem rather long: head cylindric.

On dead leaves of *Dioscorea*: Argentina (Tucuman, Parque Roca, April 15, 1906, leg. Spegazzini, Inst. Speg. n. 19270).

Spores $6-8 \times 3-4\mu$, white, ellipsoid, with a large gutta (Speg.).

Basidia $30 \times 8-9\mu$, with 4 short sterigmata (Speg.): cystidia none: hymenium not thickening.

Hyphae $3-12\mu$ wide, monomitic, *without clamps*, thin-walled, long-celled, scarcely constricted at the broad, thin septa: on the surface of the stem $3-6\mu$ wide and distinctly agglutinated: caulocystidia $-30 \times 5-10\mu$, somewhat conical to clavate, thin-walled, scattered: without crystals.

The type-collection has only four slender fruit-bodies, that have become rather mouldy. I could find no spores, but a pencil-sketch by Spegazzini clearly shows a large gutta in an ellipsoid spore, as well as the small basidia, which I found to measure $25-30 \times 7-8\mu$. The other microscopic details I have added. The fruit bodies were superficial and did not have sclerotia, as described by Spegazzini, though there were numerous small black spots with pale border on the leaf. And this leaf belongs to a species of *Dioscorea*, not to *Bignonia* as given by Spegazzini, and this, too, may have been a *lapsus calami* for *Bauhinia*. The habitat, together with the absence of clamps, should enable one to recognize the species. It may be related to *P. oleae*.

P. typhuloides (Pk.) Burt

U.S. Dept. Agr. Myc. Coll., s.n. (Colorado, Telluride, c. 2,700 m. alt., Sept. 1, 1900, leg. C. G. Shear, on dead stems of *Epilobium angustifolium*): white: spores $6-9 \times 3-3.5 \mu$: hyphae -10μ wide, the larger ones with walls $0.5-1 \mu$ thick, subgelatinous, tough, the cells often of unequal width, clamped.

The range of this species would appear to be that of the *Epilobium*, though possibly confined to its montane and boreal range.

PISTILLINA Quél.

P. calyx Heim

Mém. Soc. Hist. Nat. Afr. Nord II, 1949, 151, f. 3.

This species must be excluded. The pileate fruit-body with sterile upper surface shows that it is a minute Marasmioid fungus.

THE PHYSALACRIA-SUBSERIES HORMOMITARIA,
PHYSALACRIA, AND PSEUDOTYPHULA

I placed *Physalacria* and *Hormomitaria* in the *Clavariadelphus*-series because of their unbranched fruit-bodies of limited growth, their aguttate spores, and their 'epiphytic', non-humicolous, habitat. Neither has the ordinary clavate form of fruit-body, but this occurs in the new genus *Pseudotyphula*, which I describe here from dried collections from West Africa. The fruit-bodies of *Physalacria* appear to be ageotropic, while those of *Hormomitaria* are positively geotropic, or inverted. It was not possible to ascertain certainly the direction of growth in *Pseudotyphula*, but the stalks of the fruit-bodies appear to have been deflexed. The characters which relate the three genera are the oleocystidia, the more or less clavate and flexuous caulocystidia, the small and narrow basidia and spores, and the acerose young basidia: However, there are exceptions, as follows:

1. Oleocystidia are absent from *P. stilboidea*, which has thick-walled cystidia, but otherwise agrees closely with *Physalacria*.
2. Oleocystidia are lacking from *H. albidula*, which, as here described, is in other respects close to *H. sulphurea*.

Singer (1949) places *Physalacria* in the Agaricaceae, and I emphasized its marasmioid affinities in my monograph (p. 97). The new genus, *Pseudotyphula*, seems emphatically to prove the clavarioid affinity of the subseries. Each of the two species of *Pseudotyphula* is known only from a single, but copious, collection from West Africa. It is to be hoped that other forms of the alliance may be discovered which will throw more light on the affinity of the group. Compare, also, *Mucronella flava*, described in this paper.

KEY TO THE GENERA OF THE PHYSALACRIA-SUBSERIES

- Fertile head capitate or bullate, inflated and hollow, rarely conical (*P. andina*), ageotropic: oleocystidia generally appendaged, rarely absent and replaced by thick-walled cystidia (*P. stilboidea*): hyphae not secondarily septate: spores often much longer than wide *Physalacria*
- Fertile head conical or cylindric, solid, waxy to toughly subgelatinous: spores at most twice as long as wide.
- 12 mm. long, downpointing, conical, with very short stem; hymenium sharply delimited from the stem by a zone of cystidia or sterile basidia: hyphae secondarily septate *Hormomitaria*
- Larger, (? deflexed), conical or cylindric, the stems —10 mm. long: hymenium not so delimited: hyphae not secondarily septate *Pseudotyphula*

HORMOMITARIA Corner

H. albidula sp. nov.

Fig. 3.

Usque 2.5 mm. longa, candida, dein crenea, ceracea, dense gregaria, inversa: capitula —2 × 0.4–0.55 mm., conica: stipite 0.2–0.35 × 0.1 mm., subpuberulo: sporis 3.5–4 × 2.5–3 μ, late ellipsoideis, obtusis, aguttatis: basidiis 10–16 × 4–4.7 μ, juventute acerosis, tetrasporis: oleocystidiis deficientibus: hymenio non incrassato: basi capitulae sterili, e basidiis sterilibus instructo: caulocystidiis —20 × 2.5–6 μ, subclavatis v. subventricosis, laevibus: hyphis 2.5–8.5 μ, fibuligeris, ordine secundo septatis septis 6–70 μ distantibus, intus oleaginosus, tenuiter tunicatis: crystallis tetrahedricis —12 μ latis copiosis, praecipue in contextu stipitis: ad lignum emortuum, Brasilia (Rio de Janeiro, Corcovado, c. 500 m. alt., Nov. 28, 1948, Corner 769).

—2.5 mm. high, *inverted, conical, shortly stipitate, densely gregarious*, not fasciculate, waxy, solid, *white, then cream*: head —2 × 0.4–0.55 mm. wide below, 50–100 μ wide at the acute apex, sterile at the base: stem 0.2–0.35 × 0.1 mm., subpuberulous.

On rotten wood in the forest: Brazil (Rio de Janeiro, Corcovado, c. 500 m. alt., Nov. 28, 1948, Corner 769).

Spores 3.5–4 × 2.5–3 μ, white, smooth, broadly ellipsoid, blunt, aguttate, thin-walled, the apiculus minute.

Basidia 10–16 × 4–4.7 μ, acerosa when young, clamped: sterigmata 4, 3 μ long.

Hymenium not thickening: *oleocystidia absent: the base of the head sterile*, with rather loosely arranged sterile basidia, hyaline, scarcely modified into cystidia.

Caulocystidia —20 × 2.5–6 μ, subcylindric, subclavate or subventricose, with thin or slightly thickened walls, divergent, loose, crowded at the stem-apex and with transitions to the sterile basidia, smooth.

Hyphae 2.5–8.5 μ wide, clamped, but many of the cells becoming secondarily septate with the septa at intervals of 6–70 μ, not forming moniliform rows,

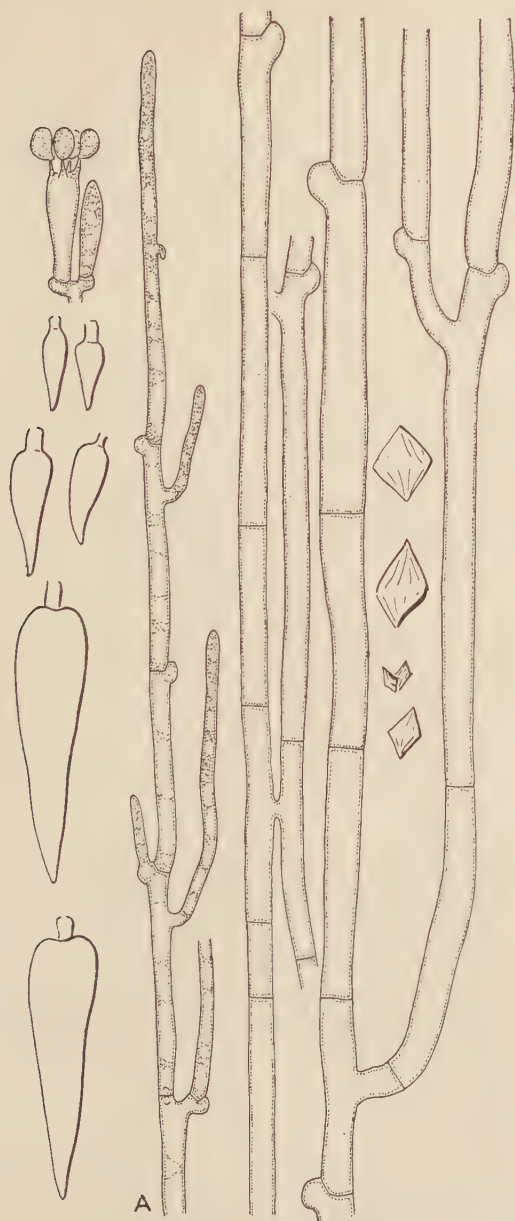


FIG. 3. *Hormomitaria albidula* sp. nov.: fruit-bodies, $\times 18$: spores, basidia, and hyphae, $\times 1,000$: A, a hyphal tip from the apex of the fruit-body (Corner 769)

thin-walled, with oily contents, not agglutinated at the surface: crystals — 12μ wide, tetrahedral, abundant in the flesh, particularly in the stem: hyphal tips $1.5\text{--}2.5\mu$ wide.

This species differs from *H. sulphurea* in the smaller, white fruit-bodies, smaller spores, narrower hyphae not forming moniliform rows of cells on becoming secondarily septate, shorter caulocystidia, and absence of oleocystidia. The habit and general construction of the fruit-body is the same. Compare *Mucronella*.

PHYSALACRIA Pk.

P. aggregata Martin et Baker

Corner 789, Brazil (Rio de Janeiro, Corcovado, *c.* 500 m. alt., Nov. 20, 1948, on the underside of dead sticks in the forest): $0.5\text{--}1.2$ mm. high, white, gregarious: head $0.3\text{--}0.8$ mm. wide, subglobose, with sterile base, hollow: stem $0.2\text{--}0.4$ mm. $\times 70\text{--}150\mu$, puberulous: spores $4.5\text{--}5.5 \times 2.3\text{--}3\mu$, subcylindric, blunt: basidia $12\text{--}15 \times 3.5\text{--}4\mu$, subacrose when young, with 4 sterigmata $2.5\text{--}3\mu$ long: oleocystidia $35\text{--}50 \times 7\text{--}13\mu$, fusiform-ventricose, with a neck $4\text{--}8 \times 3.5\text{--}5.5\mu$, resin-capped, slightly thick-walled in the neck: sterile basidia at the base of the head clavate, $12\text{--}18 \times 7\text{--}12\mu$: caulocystidia as reduced oleocystidia, abundant at the base of the stem: hyphae $1.5\text{--}3.5\mu$ wide, uninflated, clamped, thin-walled, — 7μ wide on the surface of the stem and short-celled and subagglutinated.

This collection has slightly smaller fruit-bodies and narrower spores than Martin and Baker describe, but the sterile base is characteristic.

P. andina Pat.

Venezuela (Chardon and Toro n. 1404, Pelasi-Cacaquita Road, Est. Miranda, July 11, 1932): spores $9\text{--}12 \times 3\text{--}3.5\mu$: basidia?: oleocystidia typical: hyphae clamped, short-celled ($4\text{--}10\mu$ wide) and agglutinated on the stem-surface and tuberculate on the outer side with minute processes — $2.5 \times 1\mu$.

This collection was identified by Professor G. W. Martin, with whom I agree, though I was unable to check the number of spores on the basidium. Compare *P. concinna*.

P. concinna Syd.

Fig. 4.

The following four collections from Florida, loaned to me from U.S. Department of Agriculture Mycological Collections, must be identified with *P. concinna* in view of the short spores and the very abundant cystidioles. The collections have the papillate surface-hyphae on the stem, as in *P. andina*, of which they may be regarded as the tetrasporous state, but the curious cystidioles have not been reported for this species. Cystidioles are reported for *P.*

orinocensis, which appears to have much larger fruit-bodies and shorter spores: its stem-hyphae are not known. The three species suggest a close alliance.

—1 mm. high, solitary or gregarious, rarely in small clusters, white, superficial: head —0.6 × 0.25–0.35 mm., ovoid-conic, very minutely hispid when dried: stem —0.5 mm. × 80–120 μ , slightly brownish at the base, minutely pruinose when dried.

On dead petioles, leaves, and twigs: Florida (Highlands Hammock, on petioles and leaflets of *Rhus copallina*, March 15, 1937, leg. C. G. S. n. 927;

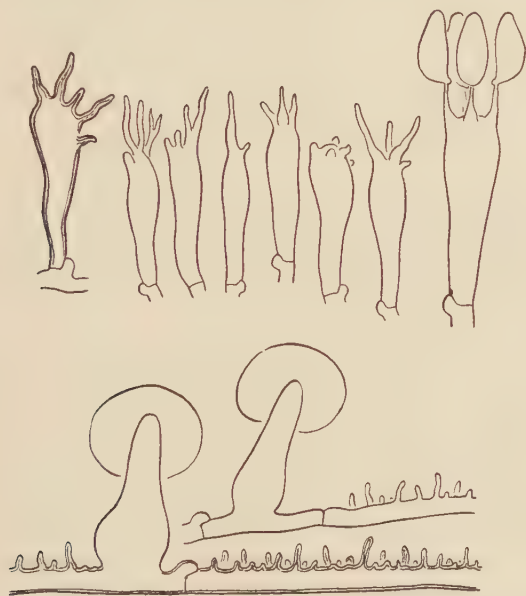


FIG. 4. *Physalacria concinna* Syd.: a basidium with nearly mature spores, cystidioles (that on the left being from the base of the head), and stem-hyphae with oleocystidia, $\times 1,000$ (Florida, Highlands Hammock, leg. C.G.S., March 15, 1937)

Dade City, on twigs of *Salix*, April 1938, leg. C.G.S. s.n.: Rock Spa, on twigs of *Tilia*, April 3, 1944, leg. C.G.S. s.n.: Winter Park, on a Lauraceous twig, December 10, 1942, leg. C.G.S. s.n.).

Spores 8–10.5 × 3.5–5 μ , white, smooth, ellipsoid to subcylindric, with a small apiculus.

Basidia 22–30 × 7–8.5 μ , clavate, 2.5–3.5 μ wide at the base: sterigmata 4, stout, 5–6.5 μ long.

Cystidioles 18–23 × 3.5–6 μ , very abundant, subclavate, set with 1–6 sterigma-like processes —8 μ long, irregularly arranged at or below the apex.

Oleocystidia 35–75 × 6–13 μ , ventricose-lanceolate, with a short obtuse or subcapitate neck embedded in a globose oily-resinous mass 10–17 μ wide, thin-walled or with firm walls, usually with a long base, projecting to 30 μ : transitions to cystidioles few.

Hymenium not thickening, composed mainly of cystidioles, the basidia appearing very scattered and apparently late in developing: hyphae of the head $2-4.5\mu$ wide, thin-walled (? subgelatinous), not inflated, branched at a wide angle: base of the head apparently not differentiated as a sterile zone, though some of the cystidioles in this part rather larger and slightly thick-walled.

Stem composed of compact, longitudinal hyphae $2-7\mu$ wide, clamped, the cells $20-110\mu$ long, the walls firm or slightly thickened: superficial hyphae $2-5\mu$ wide, the walls thin or thickened to 2μ , scarcely agglutinated, densely papillate on the outer side with processes $-5 \times 1-1.5\mu$, rarely -15μ long: caulocystidia as short oleocystidia, $-30 \times 12\mu$, thin-walled, ventricose to subcylindric, colourless.

PSEUDOTYPHULA gen. nov.

Receptaculis simplicibus, gregariis v. fasciculatis, (? deflexis), cylindricis v. elongato-conicis et attenuatis, gracilibus, stipitatis: capitulis plenis, a stipite vix distinctis: hymenio non incrassato: sporis, basidiis, oleocystidiis et caulocystidiis ut in *Physalacria*: hyphis monomitricis, fibuligeris, haud ordine secundo septatis, in stipite crasse tuni-catis, in capitulo subgelatinosis: ad lignum emortuum, Africa Occ., 2 spp., typus *P. ochracea* sp. nov.

Fruit-bodies *simple*, gregarious or fasciculate, (? deflexed), *cylindric or elongate-conical* and tapering, slender, *stalked*: *fertile heads solid*, not distinctly marked off from the stem but with a region of gradual transition: hymenium not thickening: spores, basidia, oleocystidia and caulocystidia as in *Physalacria*: hyphae monomitic, clamped, not secondarily septate, becoming thick-walled in the stem, subgelatinous in the head, on dead wood, West Africa, 2 spp.

KEY TO THE SPECIES OF PSEUDOTYPHULA

- 6 cm. long, solitary or gregarious: head long, cylindric: stem $0.3-0.4$ mm. wide: oleocystidia with subcylindric appendage. *P. ochracea*
- 2 cm. long, caespitose: head conical, rather short: stem $0.1-0.2$ mm. wide: oleocystidia with subcapitate appendage. *P. tenuipes*

P. ochracea sp. nov.

Lloyd Myc. Notes 75, 1925, 1359, f. 3216 (ut *Pterula Winkleriana*).

Usque 6 cm. longa, solitaria v. gregaria, cylindrica, sicca claro-ochracea: stipite $-10 \times 0.3-0.4$ mm., minute pruinoso, e disco mycelio pallide ochraceo pusillo oriundo: carne solida, sicca dura: sporis $4.5-5.5 \times 2.5\mu$: basidiis tetrasporis: oleocystidiis $30-50 \times 8-11\mu$, clavato-ventricosis, ad apicem processu subcylindrico $6-9\mu$ longo appendiculato, haud projicientibus: caulocystidiis $-35 \times 4-10\mu$, clavatis v. subventricosis, levibus: hyphis $2.5-10\mu$ latis: ad lignum emortuum, Belgian Congo (Lloyd Cat. No. 32725, leg. H. Vanderyst, Sept. 1922, pr. Ipama).

—6 cm. long, solitary or gregarious, *cylindric, clear ochraceous when dried*, the fertile head $0.4-0.7$ mm. wide: stem $-10 \times 0.3-0.4$ mm. wide, finely pruinose, *arising from a minute, villous, pale ochraceous, mycelial patch*, those

of adjacent primordia often confluent: hymenium smooth, not sharply delimited: flesh drying hard, *solid*, (? toughly subgelatinous, when fresh).

On dead wood: Belgian Congo.

Spores $4.5-5.5 \times 2.5 \mu$, white, smooth, thin-walled, ellipsoid to subamygdaliform, blunt.

Basidia $20-25 \times 3.5-4 \mu$, narrow: sterigmata 4; hymenium not thickening: subhymenial hyphae $2-3.5 \mu$ wide, much interwoven, not subgelatinous.

Oleocystidia $30-50 \times 8-11 \mu$, clavate-ventricose with a short subcylindric appendage $6-9 \mu$ long, immersed in the oily resinous excretion, thin-walled, immersed, the apex just reaching the surface of the hymenium, the base long and narrow.

Caulocystidia — $35 \times 4-10 \mu$, more or less clavate to subventricose, thin- or slightly thick-walled, smooth, not encrusted, colourless, often flexuous and decumbent, crowded at the base of the stem: with gradual transition to the hymenium.

Hyphae $2.5-10 \mu$ wide, clamped, longitudinal, with firm and distinctly thickened walls (-1μ) in the stem, not agglutinated at the surface: with toughly gelatinous, more or less agglutinated walls in the fertile head, and with much fine granular matter with acicular crystals: the cells of the hyphae $25-150 \mu$ long.

Mycelial hyphae $2-4 \mu$ wide, clamped, with rather thick walls, ending in caulocystidia all over the surface of the mycelial patch round the base of the stem.

***P. tenuipes* (Lloyd) comb. nov.**

Basinym: *Mucronella tenuipes* Lloyd, Myc. Notes 55, 1918, 789, f. 1187.

—2 cm. high, simple, *densely caespitose*: stem $-10 \times 0.1-0.25$ mm., cylindric, minutely puberulous, drying pallid ochraceous drab: *head* $-10 \times 0.5-1$ mm., *conical*, gradually tapered to the acute apex, drying dark ochraceous: (? waxy-fleshy, when fresh).

On dead wood: Angola (Lloyd Cat. No. 26654, Loanda, at Sera-Subluali, Chilnango, leg. J. Gossweiler n. 88, March 1917).

Spores $3.5 \times 2.5 \mu$, white, smooth, ellipsoid.

Hymenium not thickening, with much fine granular matter obscuring the small basidia: no zone of sterile cells at the stem-apex.

Oleocystidia $35-50 \times 10-16 \mu$, the subcapitate apex $5-6 \mu$ wide, thin-walled, ventricose-capitate.

Caulocystidia — $50 \times 3-8 \mu$, cylindric to subclavate, thin-walled, not encrusted, often flexuous, colourless, forming a loose, disrupted palisade.

Hyphae $3-10 \mu$ wide in the stem, -15μ wide in the head, clamped, thin-walled, or with slightly thickened and agglutinated walls in the stem.

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Experimental and Analytical Studies of Pteridophytes XXII. Morphogenesis in *Selaginella Willdenovii* Baker

I. Preliminary Morphological Analysis¹

BY

F. CUSICK

(Botany Department, The University, Aberdeen)

With Plate XX and three Figures in the Text

ABSTRACT

Changes of configuration at the shoot apex of *Selaginella Willdenovii* during the formation of branches and leaves are described and discussed in terms of growth.

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I. INTRODUCTION

THE genus *Selaginella* differs from seed plants and ferns in the inherent dorsiventrality of most of its species, in the microphyllous nature of its leaves, in its terminal branching, and in its possession of angle meristems, organs which are capable of development, under different conditions, either as rhizophores or as shoots. In investigations to be described the author has re-examined the meristematic regions of *Selaginella Willdenovii* by morphological and experimental techniques and has attempted to state the major problems of shoot morphogenesis in this species. The work comprises (1) a preliminary morphological analysis, (2) experimental studies on angle meristems, (3) experimental studies on shoot apices; the present paper deals with the first of these.

¹ The work reported here was carried out in the Department of Cryptogamic Botany University of Manchester and submitted for publication from the Botany Department, University College of Wales, Aberystwyth.

With the genus *Selaginella* there is considerable diversity in apical structure; among dorsiventral species *S. Lyallii* has a group of small equivalent apical cells (Russow, 1873; Bruchmann, 1909), but most species show a regular segmentation from one or more large apical cells (Treub, 1874; Wand, 1914; Williams, 1931). In accounts of species possessing well-defined apical cells, changes in cell shape and cell arrangement are frequently mentioned and it can be concluded that there is no necessary connexion between the pattern of cells at the shoot apex and the course of leaf inception or axial branching. But, descriptions of apical cells, given without reference to the subjacent regions, are of so little help in studying morphogenesis that little use can be made of much of the earlier work.

The dorsiventrality of *Selaginella* has already been the subject of some experimental investigation. In *S. hortensis*, as in other dorsiventral species of *Selaginella*, the morphologically dorsal side receives most illumination and bears the smaller leaves; Hofmeister (1868) found that this condition persisted in plants grown in darkness. Pfeffer (1871) inverted branches of *S. Kraussiana* between glass plates, in this way altering their orientation to light and to gravity. Neither the orientation of the dorsal and ventral surfaces of the shoot nor the size proportion of the dorsal and ventral leaves was changed in the new growth. Goebel (1900) stated that 'in *S. sanguinolenta* alone of its genus anisophylly is not constant but appears to be under the control of external factors'; later (1915) he showed that in *S. caracana* anisophylly is first induced by light but that the plane of dorsiventrality is not affected by the direction of the light. Meanwhile Fries (1911) had shown that in *S. Martensii* and *S. Willdenovii* the orientation of dorsal and ventral-angle shoots is independent of external factors.

The internal regulation of morphogenesis has also been studied experimentally in angle meristems. In the presence of the shoot apex, angle meristems develop as rhizophores or remain dormant; in the absence of the main apex they develop as main shoots. Behrens (1897), working with *S. inaequalifolia* and *S. uncinata*, found that angle meristems separated from the axial apex formed shoots, however small the size of the twig on which they were isolated; this indicated that the 'shoot reaction' was not due to nutritional factors, as had previously been maintained. Williams (1937) applied *b*-indole-acetic acid to isolated branchings of *S. Lobbii* and *S. Martensii*. Angle meristems which were undeveloped at the start of the experiment grew out as rhizophores, as they normally would have done on intact plants. Controls, isolated without indole-acetic acid, produced shoots. Williams concludes that these results 'do not prove that the correlating influence normally exercised by the shoot apex is in the nature of a hormone mechanism but they lend strong support to this view'. Earlier Williams (1931) had shown that in *S. grandis* the stem apex is more active than the angle meristem; that the activities of cone apices and angle meristems are relatively equal, and that the activity of the angle meristems appears to be correlated to their position in relation to the apex of the shoot. The 'apex' whose morphogenetic activity is so convincingly

demonstrated in all these experiments is the macroscopic apex, some half an inch or more in length; but within this region many of the characteristics of the mature shoot are already present—the greater part of morphogenesis has already occurred.

The description of the meristematic regions of a plant *in terms of growth* is of threefold value: it expresses the instability and the cyclic changes which are their most striking feature, it contributes towards an explanation of mature form, and it is capable of further analysis at the physiological level. Where growth takes place round a central meristematic region of radial symmetry, morphogenetic pattern can be stated concisely, and fairly accurately, by means of contact leaves and plastochrone ratios; in *Selaginella Willdenovii*, where the shoot apex itself shows bilateral and dorsiventral growth, a more detailed verbal description is necessary.

2. MATERIALS AND METHODS

Materials for the study of the vegetative shoot of *Selaginella Willdenovii* were taken from a stock culture growing in a moist frame in a warm greenhouse. Fertile fronds were obtained from the Royal Botanic Gardens, Kew. Apices were partially dissected to help orientation and sectioned by the paraffin method. For organographic work, transverse sections cut from the apex backwards are the most useful as they permit accurate reconstruction of the meristematic regions in drawings and models; thick sections (24–40 microns) are preferable to thin ones because they allow more of the convex outer surface of the apex to be seen at once and because there is less inaccuracy when drawings of successive sections are superimposed. Series of thinner sections (10–15 microns), cut in various planes, have been used for the study of internal structure.

3. TERMINOLOGY

The shoot system of *Selaginella Willdenovii* consists of an aerial *axis* of indefinite growth, the apex of which branches periodically; at successive bifurcations of this axis, right and left shanks alternately develop as *branches* which are characterized by relatively quick growth and frequent sub-branching of limited duration, and, as sub-branching proceeds, by marked anisophylly. With the exception of the angle meristems which are formed at bifurcations of the axis, all branchings of the shoot system take place in the same plane (Text-fig. 1). The major surfaces of the plant which are thus formed will be referred to as *dorsal* and *ventral*, these terms being used in a purely morphological sense, without any implication of 'upper' and 'lower': the dorsal surface is that which is directed towards the light and which, in anisophyllous parts of the shoot, bears the smaller leaves. The marginal regions which separate the dorsal and ventral surfaces of the shoot will be referred to as the *lateral* surfaces or as the *sides* of the shoot. The growth of shoot and shoot apex can thus be described with reference to three planes or axes intersecting at right angles: the *longitudinal* axis, the *lateral* axis (which lies in the

plane of the branchings), and the *dorsiventral* axis which lies in the plane of dorsiventrality.

4. THE APEX OF THE AXIS

The adult axis of *Selaginella Willdenovii* tapers sharply at the distal end and then terminates bluntly. Across the extremity is a laterally orientated band of



TEXT-FIG. 1. Distal part of a shoot of *Selaginella Willdenovii* Baker. A, in dorsal view; B, the region of the axial apex in ventral view. The lateral plane of the shoot is in the plane of the page, the dorsiventral plane at right angles to it. Ventral-angle leaves in solid black; *a*, axis; *aa*, axial apex; *b*, branch; *db*, distal branchlets; *dl*, dorsilateral leaves; *ll*, lateral leaves; *vl*, ventrilateral leaves; *val*, ventral-angle leaf; *dam*, dorsal-angle meristem. ($\times 2$.)

large, wedge-shaped cells. Barclay (1931), confining his attention to primary sporeling shoots of *S. Willdenovii*, has described a single tetrahedral apical cell; no sporeling material has been examined by the present author.

In a surface end-on view of the apex of a well-grown plant (Pl. XX, figs. 1-5) the cells of the lateral band appear rectangular with their longer dimension in the dorsiventral plane of the shoot. Sections cut in this plane show the cells as approximately equilateral triangles (Pl. XX, Fig. 7 A); sections cut in the

lateral plane of the shoot show them with rectangular or triangular outline, the conspicuous long axes of the cells directed into the interior of the shoot (Pl. XX, Fig. 7, B). The constituent cells of the apical band have no individual permanency as such; a continual reduction in size takes place at the lateral extremities of the band where segmentation of its cells adds to the sub-apical zone. This loss is offset by the division, along the lateral axis, of the remaining cells of the band (the new cell walls formed in the course of these divisions are in the dorsiventral plane of the shoot); there is actually a steady increase in the length of the band and in the number of its component cells up to the time of branching. The apical cells also segment in the dorsiventral plane; in surface view, these divisions appear to be very unequal, but the volumes of the two cells resulting from such a division are probably not dissimilar. Periclinal walls are not formed in the apical band; in the subjacent regions, cell divisions occur in all planes and cell size is markedly reduced. Within the apical band itself there is a gradation in cell size, the smallest cells being at the opposite sides; the largest cells are asymmetrically placed and nearer to that side which is above the last-formed branch.

The shoot of *S. Willdenovii* is a *dichopodial* system, in Troll's terminology (1937, 1949). Branching is brought about by the segmentation, without other compensating developments, of the cells which occupy an approximately median position in the band; in this way the two ends of the band become separated by a small-celled inter-apical region (Pl. XX, Figs. 2-4). The curvature of the shoot tip prevents an exact comparison of the numbers of cells in the two 'new' apices, but the one on the side above the previous branch is of greater bulk, consistently including the larger cells from the parent apical band: this apex continues the axis; the opposite side, with the smaller cells, becomes the new branch. In the apex of the axis the largest apical cells are now (through the manner of bifurcation) nearer to the inter-apical region, i.e. towards the new branch; conditions at its next bifurcation will therefore present a mirror image of those which have been described. Because the two new apices at a dichotomy are formed simultaneously, their products might be regarded as being fundamentally equivalent; in the course of their development, however, alternate shanks at successive bifurcations undergo stronger development and together constitute the axis. But the dichotomies of the apical band are unequal, and the larger-celled portion is continuously the apex of the axis; it is therefore possible to regard the smaller portions of apical tissue which are detached at dichotomies as lateral growth-centres which are formed alternately left and right in the axial apical band; this point of view, which is implicit in the present terminology of 'axis' and 'branch', is preferable in so far as it draws attention to the presence of differential development at the extreme tip of the shoot. Although the apical band is laterally elongated throughout these periodic changes, the sub-apical regions are approximately circular in cross-section except during a short period after the dichotomy of the apical band: before the new apices, by their further growth, have projected separately, the sub-apical regions are laterally elongated (Pl. XX,

Figs. 3, 4) (this shape is maintained throughout maturation); and immediately above the junction of the branch, the sub-apical regions of the axis are elongated in the dorsiventral plane (Pl. XX, Figs. 1, 2). Dichotomy of the apical band usually takes place when the axis has grown only some 200 microns beyond the junction of the previous branch.

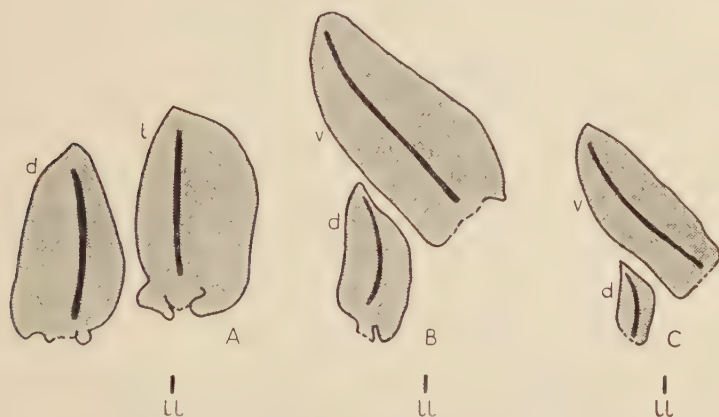
5. THE INCEPTION OF LEAVES ON THE AXIS

The inception of leaves on the axis takes place in the sub-apical zone and is confined, except at branchings, to lateral and dorsilateral positions. On each side of the shoot dorsilateral and lateral leaves alternate in acropetal succession, forming a leaf series (Pl. XX, Fig. 6). Primordia of the same series are formed in close proximity to each other; opposite series are separated by the untenanted dorsal and ventral surfaces of the shoot (Pl. XX, Figs. 1-6). Elongation of the axis brings about the separation of the leaves, and the final spacing between members of opposite series is very irregular; a lateral and a dorsilateral leaf of opposite series may occur at the same level (an approximately decussate arrangement); pairs of dorsilateral leaves may alternate with pairs of lateral leaves; or any intermediate relationship may occur. Changes between the different patterns may take place abruptly and frequently in a particular shoot (that illustrated in Text-fig. 1, for instance, though chosen at random, shows this to some extent). As the fully elongated axis is characteristically straight, these irregularities must already be present in the meristematic regions; they are sometimes visible there, but the small size-ratio of leaf to axis (the youngest leaf primordium occupies one-eighth to one-tenth of the axial circumference) does not permit accurate measurement. Because the leaf primordia of different series show no constant spatial relationship, it is unlikely that there is appreciable physiological interaction between them; and this may be due to the remoteness from each other of the two series on the axial apex.

The tangential extension of the leaves during their development takes place by the inclusion of tissue from the neighbouring dorsal or ventral surface of the shoot (these surfaces are themselves growing rapidly at this time and the untenanted space is considerable, particularly on the ventral surface, throughout development); there is no interposing of lateral and dorsilateral leaves of the same series. This kind of growth results in the asymmetry of the leaves: that leaf margin which has been extending on to the adjacent surface of the axis comes to be farther away from the originally median midrib (Text-fig. 2a). This account of a leaf asymmetry differs from that of Goebel (1913, 1927) and Troll (1937). Troll, writing of the genus as a whole, describes the outwardly (i.e. laterally) directed part of the dorsal leaf (dorsilateral in the present terminology) as larger than the inner (i.e. dorsal) part; this type of asymmetry occurs on the branches but not on the axis of *S. Willdenovii* (see section 7).

At all bifurcations of the shoot system there is an additional leaf, the *ventral-angle leaf* (Text-fig. 1). Shortly after apical dichotomy the more rapid growth-rate of the new apices results in their projection as bulges; the

inter-apical region now appears as a depression between them, rather more close to the branch than to the axis. A further bulge now appears on the ventral surface of the shoot, just below the inter-apical depression; this is the primordium of the ventral-angle leaf. On the axis it grows to approximately twice the size of the neighbouring leaves (dorsilateral and lateral leaves are here sub-equal), and its expanded 'ear' shape contrasts with their triangular form. A *dorsal-angle leaf* in the corresponding dorsal position was present at less than 5 per cent. of axial bifurcations in the Manchester materials on which



TEXT-FIG. 2. Adjacent leaves, abaxial surface uppermost, A, on axis; B, on major shank of branch; C, on distal branchlet. *d*, dorsilateral leaf; *l*, lateral leaf; *v*, ventralateral leaf; midribs in solid black; *ll*, indicates the middle of the lateral surface. ($\times 8$.)

the present observations are based; but transplants of these materials now growing at Aberystwyth have dorsal-angle leaves at 30 per cent. of their axial bifurcations. The presence or absence of the dorsal-angle leaf seems, therefore, to be controlled by external factors; the control is probably indirect and in this connexion the effects of humidity deserve attention in the future. The dorsal-angle leaf is of similar size and form to the neighbouring dorsilateral leaves. In the course of dissections, several early-stage dorsal-angle leaves have been found and these have been much smaller than the corresponding ventral-angle leaf; sometimes smaller even than later-formed lateral and dorsilateral leaves. It is possible that the projection of tissue marked *x* in Pl. XX, Fig. 2, is an aborted dorsal-angle leaf. No preparations showing it at the primordial stage have been found, nor has it yet been seen at bifurcations of branches.

At the time when the ventral-angle leaf appears, further segmentation has reduced cell size in the inter-apical region. This process, however, does not involve a group of cells above the ventral-angle leaf which later increase in size whilst remaining meristematic and form a *ventral-angle meristem* (Pl. XX, Figs. 4, 5, and 1, 2). A *dorsal-angle meristem* is also formed in a similar position on the dorsal surface of the shoot, irrespective of the presence of a dorsal-angle

leaf; its inception is probably later than that of the ventral-angle meristem because several dichotomizing axes which already show a meristem towards the ventral surface do not show one dorsally. For *S. grandis* Williams (1931) has stated that dorsal- and ventral-angle meristems probably result from the division of a single meristem. A dorsal- and a ventral-angle meristem occur at all bifurcations of the axis of *S. Willdenovii*, but neither is present at bifurcations of branches (Pl. XX, Fig. 8). The subsequent development of the angle meristems will be dealt with in a later paper.

The first member of a leaf series to appear on the inner side of each shank at a bifurcation (i.e. in the inter-apical region) is always dorsilateral in position; it is followed by a lateral leaf which is between, though distal to, the dorsilateral leaf and the ventral-angle leaf; succeeding leaves follow the pattern already described, dorsilateral and lateral leaves alternating in an acropetal series. Although it is convenient to regard the ventral-angle leaf as 'additional' to the leaf series, the regular sequence of leaf inception in the inter-apical region suggests that it may be active in the determination of the next leaf positions. On the outer sides of the dichotomy, leaf formation continues without a break from the axis below (Pl. XX, Figs. 1 and 2).

6. AXIS AND BRANCH

For a short time after dichotomy the branch is in all dimensions smaller than the axis, perhaps because of its smaller apex. By the time the axis has bifurcated again, however, and until the end of its limited growth, the branch overtops the axis; it also bifurcates more frequently: while the axis subdivides once, the branch produces (average of 10 specimens) 5 sub-branch apices (cf. Text-fig. 3). Sub-branching is dichopodial throughout, but the major shanks of the branch are never overtopped in the course of their development. At the first dichotomy of the branch the major shank is usually that which is the farther away from (i.e. abaxial to) the axis: in 27 out of 137 bifurcations there was an *adaxial* major shank; and in 6 mature shoots in which it has been possible to compare apical cell size in ad- and abaxial shanks at the first dichotomy of a branch, only one showed the largest cells to be in the adaxial shank. It is possible therefore that the relative development of the two shanks is correlated with the size of their apical cells. It may be noted here that when an angle meristem develops as a shoot, the early branches, at their first dichotomy, have an adaxial major shank; a reversal of this arrangement usually occurs in the fourth or fifth branch to be produced, but the pattern is never completely stabilized (the numbers given above refer to the distal regions of adult shoots).

At all orders of sub-branching the major shank is always that which is the nearer to the major shank of the previous bifurcation (Text-fig. 1). Sub-branching is accompanied by a gradual diminution in the sizes of apices and branchlets and by a progressive increase in anisophylly; the inception of leaves, however, continues with little change the pattern already described (cf. Pl. XX, Figs. 5 and 8). Eventually growth ceases and is not normally

resumed, though proliferation can sometimes be induced by the excision of the still actively meristematic regions of the plant.

7. THE DISTAL APICES OF THE BRANCH

The apex of a distal branchlet after growth, or at least macroscopic growth, has ceased is illustrated in Pl. XX, Fig. 9. The obvious differences from the axial



TEXT-FIG. 3. The terminal regions of the axis, constructed from camera-lucida drawings of serial sections cut in the lateral plane of the shoot; all stem apices indicated by a dot; only the bases (*lb*) of the leaves are drawn. *a*, axial apex; *b1*, *b2*, *b3*, successively older branches. ($\times 27$.)

apex are in the absolute and relative sizes of the organs. The constituent cells of the apical band of the branchlet are fewer and smaller in size, and in the sub-apical zone linear dimensions are approximately one-third of those of the axis; the leaf primordia are less than proportionately reduced, the youngest occupying about one-fifth of the circumference of the stem. Because of this there is little untenanted space on the dorsal and ventral surfaces of the branchlet and leaves of the lateral rows are, in all the finer divisions of the branch, ventrilateral in position. Leaf primordia are produced in opposite pairs but successive pairs are not at right angles because the stem is laterally elongated in cross-section; leaf arrangement, therefore, is not strictly decussate. When first visible, primordia of dorsilateral and ventrilateral rows are

of approximately equal size; anisophylly is the result of differential growth at a later stage in their development.

Changes in leaf asymmetry accompany the sub-division of the branch. First in dorsilateral, then in lateral (or ventrilateral) leaves, differences in tangential growth on opposite sides of the midrib diminish (Text-fig. 2, *b* and *c*); this is probably correlated with the greater proximity of the two leaf series. During the development of the individual leaf the line of junction of leaf and stem changes from transverse to obliquely longitudinal (farthest away from the apex at the lateral margin). A detailed account of these changes is not attempted here, but it is interesting to note that, as a result of their oblique junction, the dorsal parts of dorsilateral leaves are of smaller area than the lateral parts (Text-fig. 2, *b* and *c*); this is a reversal of the condition on the axis and is in accord with Troll's generalization (see section 5). In ventrilateral leaves either part may be the larger; here compensating growth in the ventral portion of the leaf turns the whole leaf to a position at right angles to its oblique line of insertion on the stem.

8. THE APEX OF THE FERTILE CONE

In fertile shoots distal branchlets continue growth as terminal cones of sporophylls. The limited number of cone apices in which cellular configuration has been seen have each a laterally orientated band of apical cells (Pl. XX, (Fig. 10), except one with a single square-prismatic apical cell. Goebel (1927) has already stated that, although the fertile cones of dorsiventral species of the genus are apparently radially symmetrical, transverse sections of the apex are elliptical in outline. The leaf (sporophyll) primordia which are formed in the sub-apical regions are approximately equal in size to those of the axis, but the branchlet on which they are borne has dimensions which are only half those of the axis; sporophylls are formed in four vertical rows in a truly decussate arrangement, and there is no continuously untenanted surface on the apex of the cone. Sporangia develop in the axils of the sporophylls whilst these are still the youngest members of their rows. The sporophylls succeed foliage leaves without interruption of the normal sequence, but quickly successive pairs come to be produced at right angles to each other.

9. THE DIFFERENTIATION OF VASCULAR TISSUE

Below the band of apical cells there is a quick transition to a homogeneous tissue of isodiametric, meristematic cells; at a slightly lower level the centrally placed cells show marked elongation, forming a core of incipient vascular tissue which extends distally to within 3–5 cells of the apical band (cf. Wardlaw, 1944, Pl. III, Fig. 5). The first xylem to develop from the incipient vascular tissue is recognizable by the large lumina of the cells at *c.* 175 microns below the tip of the axis; in transverse section it is seen as a median row of cells in the lateral plane of the shoot. At a slightly lower level the differentiation of two further xylem bands, one dorsal and one ventral to the middle

take place. In large-shoot apices two smaller xylem bands may differentiate, again at a slightly lower level, between existing bands. (The xylem referred to is metaxylem; protoxylem, which has not been studied in detail, is only conspicuous below the junction of leaf-traces to the axial stele, though it may be present above this level.) The tissue immediately surrounding each xylem band is later organized as phloem and pericycle; meanwhile the rest of the incipient vascular tissue vacuolates into parenchyma, with the cells in characteristic longitudinal files. Concurrently, vacuolation occurs in the zone surrounding the incipient vascular core, usually beginning below the penultimate primordium of a row of leaves and about 200 microns below the shoot tip. The greater growth-rate of the vacuolating tissue in the cortical and intrastelar regions leads to the formation of lacunae round each vascular band, as has earlier been observed by Wardlaw (1925); the lacunae are first visible some 500 microns below the apex, but the characteristic periclinal divisions which precede the formation of the trabeculae can be seen above this.

Three vascular bands are present in the major shanks of the branch, only one in the finer sub-branches. Where a branchlet becomes fertile, the vascular strand at first continues to differentiate in the lateral plane but, as sporophylls begin to be formed, the stele becomes circular in cross-section; immediately below the junction of the traces of each pair of sporophylls, however, it is elongate with the traces at opposite ends of the band. In the primary sporophyllous shoot, Barclay (1931) has described a single, laterally orientated vascular band.

10. DISCUSSION

From the foregoing description two broad facts stand out. First is the pervasive dorsiventrality of the shoot system of *S. Willdenovii*; of particular interest is the existence of this dorsiventrality in the shoot apex. Second is the degree of complexity in the form of the shoot which is attained by the differential growth of a dichotomizing leafy axis.

The form of a plant is the direct expression of its differential growth; of the complex symmetry of *S. Willdenovii* it may therefore be said that:

- i. There is more growth in the lateral plane than in the dorsiventral plane; this gives the shoot bilateral symmetry.
- ii. Within the lateral plane the growth pattern fluctuates regularly from side to side, symmetry being achieved at every second branching.
- iii. In the dorsiventral plane there is a growth gradient of permanent orientation; conditions on the ventral surface are apparently more favourable for growth, as is shown by the occurrence of the ventral-angle leaf, by the precocious development of the ventral-angle meristem (Cusick, 1949), and by the larger size of ventrolateral leaves on anisophyllous branchlets. The fact that the ventral surface of the axis has a broader expanse unoccupied by leaves than has the corresponding dorsal surface may seem to be contrary evidence; on the other hand, it may express greater lateral growth on the ventral surface.

The preponderance of lateral growth in the apical band results in all bifurcations being in a single (lateral) plane. In the sub-apical region the dorsiventral axis increases rapidly relative to the lateral axis (see contour lines in Pl. XX, Figs. 1-4), but it must be realized that on the sides of the shoot, growth now takes place by the inception of new organs and their development as leaves; it is possible that in the course of their rapid growth the leaves inhibit the lateral growth of the axis.

Previous work, reviewed in the introduction, suggests that external factors have no appreciable effect on the origin of leaves and branches though certain features, such as the formation of the dorsal-angle leaf, may be affected by the external environment. The present observations show that leaf primordia are not formed in the first available space as defined by M. and R. Snow (1931, 1933, 1935, 1947, 1948) for certain angiosperms: in *Selaginella* the broad dorsal and ventral surfaces of the axis are, except at dichotomies, devoid of appendages. The alternation of dorsilateral and lateral members of a leaf series suggests some regulation by existing leaf primordia of further leaf inception, but it seems clear that conditions are more favourable for new leaf formation in the vicinity of existing leaves than on the dorsal and ventral surfaces of the shoot. Where the two leaf series are remote from each other, as on the axial apex, there is no regular arrangement between members of opposite series; where the leaf series are close together, as on the apex of the branchlet or fertile cone, a regular phyllotaxis of decussate type is formed. These facts suggest that, in contrast to the condition found in *Dryopteris* by Wardlaw (1949, 1949a, 1950), the regulative effects of the leaves of *Selaginella* are of small extent, and that the absence of leaves from the dorsal and ventral surfaces of the axial apex does not result from an inhibitory activity of existing leaf primordia. It seems possible, therefore, that factors such as the diffusion of growth-regulating substances from the laterally elongated band of apical cells may be the determinant of leaf pattern, or that the inception of leaves is determined by the mechanical states of the various regions of the growing apex. These two hypotheses are not incompatible: mechanical stress occurs where different parts in the same plane are growing at different rates, and this might be associated with the diffusion of growth-regulators from the apical cells. Wardlaw (1948) has shown that in *Dryopteris* axillary regions are subject to maximal tangential stress; new leaf primordia are formed above the interfoliar positions where tangential stress is minimal. It is probable that the regions of highest tangential stress in *Selaginella* are the dorsal and ventral surfaces of the apex because changes in dimension are most marked in its lateral plane; if there is more growth ventrally than dorsally, the ventral surface will be under greater stress than the dorsal surface.

Because of the hypothetical nature of 'comparative' argument, an experimental analysis of the interactions of leaf primordia, and of the shoot apex and sub-apical regions, is required for the solution of these problems.

II. SUMMARY

1. The shoot system of *Selaginella Willdenovii* consists of an axis, of branches which are produced alternately left and right by bifurcation of the axis in a single (lateral) plane, and of angle meristems or their products (rhizophores or axes) which are formed, one dorsally, one ventrally, at axial bifurcations.

2. The growth of the branch is quicker, with more frequent bifurcation, than that of the axis; it is, however, of limited duration, whereas axial growth is unlimited.

3. The main features of bilateral and dorsiventral symmetry are already present in the meristematic regions. Each leafy stem has a laterally orientated band of wedge-shaped apical cells; where this is large, as in the axis, gradations in cell size are noticeable in definite relation to the phase of branching.

4. In the sub-apical region leaves develop in two series, one on each side of the shoot. Each series comprises alternating members of a lateral and a dorsilateral (in distal sub-branches dorsilateral and ventrilateral) row of leaves.

5. On the axis, leaf primordia occupy one-eighth to one-tenth of the sub-apical circumference at the time of their formation, and dorsal and ventral surfaces of the shoot are untenanted throughout development, except at bifurcations; here a ventral-angle leaf is invariably, a dorsal-angle leaf sometimes, formed. There is no regular spatial relationship between leaves of different series. These observations suggest that neither 'available space' nor inhibition by existing leaves is of great importance in determining the position of a new leaf.

6. With repeated bifurcation, the branch apices diminish in size; those of distal branchlets have linear dimensions approximately one-third of the corresponding axial dimensions; leaf primordia are not proportionately reduced and occupy one-fifth of the apical circumference at the time of their formation. There is here no continuously untenanted stem surface and the leaves are formed in obliquely decussate pairs.

7. Leaf asymmetry is largely dependent on the extent, much greater on the axis than on the branch, to which one flank of a primordium can and does encroach on the adjacent dorsal or ventral surface of the shoot.

8. There is some evidence of bilateral symmetry in the apex of the fertile cone, but the sub-apical regions are radially symmetrical; sporophylls are symmetrical and of equal size, and occur in opposite and decussate pairs.

9. There is more growth in the lateral plane of the plant than in the dorsiventral plane; both branches and leaves develop on the sides of the shoot. The balance of evidence suggests that conditions are more favourable for growth on the ventral surface than on the dorsal surface.

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EXPLANATION OF PLATE

Illustrating Dr. F. Cusick's paper on Morphogenesis in *Selaginella Willdenovii* Baker

In each figure (except Fig. 7), drawings of successive serial sections, represented by layers of wash, have been superimposed to give a relief map of an apex. All figures were drawn, by projection methods, at a magnification of $\times 1,000$ and afterwards reduced photographically to their present magnification of $\times 100$. Leaves which in life enclosed the apex or younger leaves are shown cut off immediately above their junction with the stem.

Fig. 1. Axial apex seen from above; largest cells of the apical band are towards the last formed branch (*b*); a ventral-angle leaf (*val*) and dorsal- and ventral-angle meristems (dotted) are present; dorsilateral leaves (*dl*) have developed, one on the axis and one on the branch, in the inter-apical region; on the other side of the axis, a dorsilateral (*dr*) and a lateral (*lr*) row of leaves are present. Sections 40 microns thick.

Fig. 2. Axial apex seen from above, shortly before dichotomy; largest cells of the apical band towards the last-formed branch (*b*); dorsilateral and lateral leaf rows (*dr* and *lr*) present on axis and branch; a ventral-angle leaf (*val*) and a possible aborted dorsal-angle leaf (*x*), and dorsal- and ventral-angle meristems (dotted) are present (dorsal and ventral surfaces are otherwise unoccupied in this and other axes). Sections 40 microns thick.

Fig. 3. Axial apex shortly after dichotomy; inter-apical region without visible differentiation of angle leaves or angle meristems; a dorsilateral row (*dr*) and a lateral row (*lr*) of leaves, comprising a leaf series, are present at each side of the stem. Sections 40 microns thick.

Fig. 4. Axial apex, from above, at a slightly later stage than that illustrated in Fig. 3. The primordium of the ventral-angle leaf (*val*) is visible and a group of large cells (*lc*) in the inter-apical region is possibly an early-stage ventral-angle meristem; the ventral-angle leaf of the preceding dichotomy (*VAL*) is also shown. Sections 24 microns thick.

Fig. 5. Axial apex with last-formed branch (*bx*) and previous branch (*b2*); a large ventral-angle leaf (*val*) and a ventral-angle meristem (*vam*) are present between the axis and *b2* (a dorsal-angle meristem occurred some sections below those illustrated). Sections 40 microns thick.

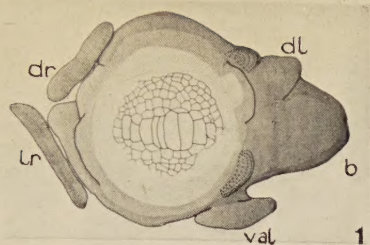
Fig. 6. The side of an axial apex showing dorsilateral- and lateral-leaf rows (*dr* and *lr*), ventral-angle leaf (*val*), dorsal- and ventral-angle meristems (dotted); a dorsilateral leaf of the opposite series is visible at *os*. Sections 6 microns thick.

Fig. 7. Axial apices seen A, in dorsiventral section, B, in lateral section; *a*, axial apex; *b*, branch apex; *lp*, leaf primordia.

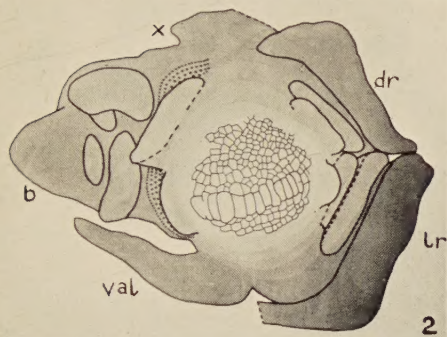
Fig. 8. The bifurcation of a sub-branch; leaf pattern resembles that on the much larger bifurcating axis (cf. Fig. 5), but no angle meristem is differentiated; leaves of each shank occur in 4 rows, 2 dorsilateral (*dr*) and 2 ventrilateral (*vr*); *val*, ventral-angle leaf. Sections 24 microns thick.

Fig. 9. Apex of a distal branchlet, showing band of apical cells, leaves in 2 dorsilateral and 2 ventrilateral rows (*dr* and *vr*); anisophylly is not apparent in the youngest pair of leaf primordia. Sections 24 microns thick.

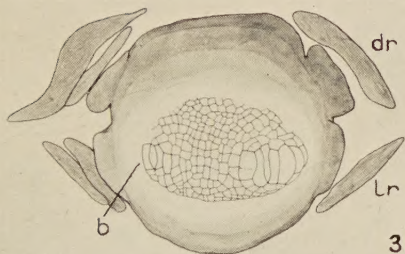
Fig. 10. Apex of a fertile cone showing the banded arrangement of the apical cells; isophyllous sporophylls (sporangia dotted) occur in opposite and decussate pairs. Sections 30 microns thick.



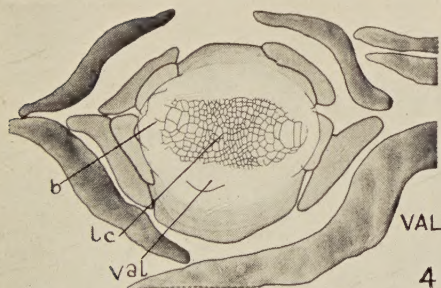
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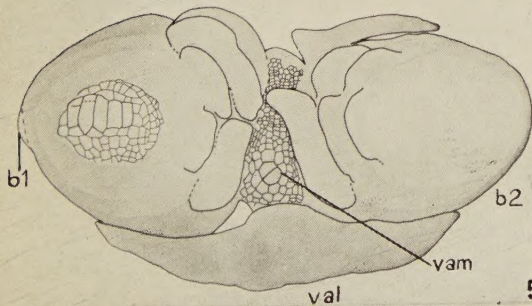
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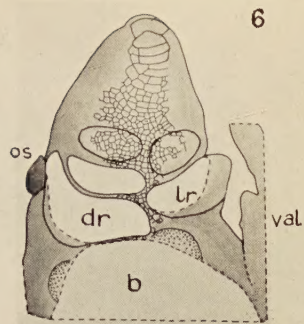
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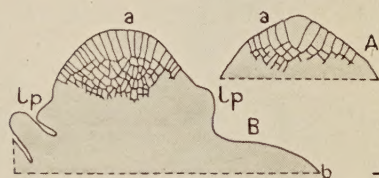
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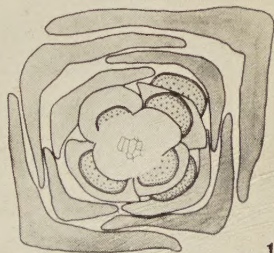
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